# Suppressive Effects of Dietary Factors on the Expression of Scavenger Receptors and Underlying Molecular Mechanisms

AI EGUCHI

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## Abbreviations

| | |
|---|---|
| ACA | 1'-Acetoxychavicol acetate |
| AcLDL | Acetylated LDL |
| AP | Activator protein |
| ATCC | American Type Culture Collection |
| AUR | Auraptene |
| CD | Cluster of differentiation |
| CLA-1 | CD36 and LIMPII analogous-1 |
| COX | Cyclooxygenase |
| CV | Cell viability |
| CXCL | CXC-chemokine ligand |
| DiI | 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindo-carbocyanine |
| DMEM | Dulbecco's modified Eagle's medium |
| DMSO | Dimethylsulfoxide |
| EGF | Epidermal growth factor |
| EGCG | (−)-Epigallocatechin-3-gallate |
| ERK | Extracellular signal-regulated kinase |
| FBS | Fetal bovine serum |
| FEEL | Fasciclin, EGF-like, laminin-type EGF-like domain-containing scavenger receptor-1,2 |
| GAPDH | Glyceraldehydes-3-phosphate dehydrogenase |
| GF | GF109293X (PKC inhibitor) |
| HDL | High-density lipoprotein |
| HBSS | Hank's balanced salt solution |
| HRP | Horseradish peroxidase |
| ICAM | Intercellular adhesion molecule |
| IFN | Interferon |
| IL | Interleukin |
| iNOS | Inducible nitric oxide synthase |
| IR | Inhibitory rate |

| | |
|---|---|
| JNK | c-Jun $NH_2$-terminal kinase |
| LDL | Low-density lipoprotein |
| LDLR | LDL receptor |
| LOX-1 | Lectin-like ox-LDL receptor-1 |
| LPS | Lipopolysaccharide |
| MAPK | Mitogen-activated protein kinase |
| MARCO | Macrophage receptor with collagenous structure |
| MCP-1 | Monocyte chemoattractant protein-1 |
| M-CSF | Macrophage colony-stimulating factor |
| MEK | MAPK kinase MAPK/ERK kinase |
| MMP | Matrix metalloproteinase |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide |
| NEG | Negative control of MAPK inhibitor (SB202474) |
| NF-κB | Nuclear factor-kappaB |
| NO | Nitric oxide |
| NOB | Nobiletin |
| $O_2^-$ | Superoxide anion |
| Ox-LDL | Oxidized LDL |
| PBS | Phosphate-buffered saline |
| PD | PD98059 (MEK1/2 inhibitor) |
| PG | Prostaglandin |
| PKC | Protein kinase C |
| PPAR | Peroxisome proliferator-activated receptor |
| ROS | Reactive oxygen species |
| RPMI | Roswell Park Memorial Institute |
| RT-PCR | Reverse transcription-polymerase chain reaction |
| SB | SB203580 (p38 MAPK inhibitor) |
| SD | Standard deviation |
| SP | SP600125 (JNK1/2 inhibitor) |
| SR | Scavenger receptor |
| SREC | SR expressed by endothelial cells |
| SR-PSOX | SR for phosphatidylserine and ox-LDL (CXCL16) |

| | |
|---|---|
| TEER | Transepithelial electrical resistance |
| TNF | Tumor necrosis factor |
| TPA | 12-*O*-Tetradechanoylphorbol-13-acetate |
| VCAM | Vascular cell adhesion molecule |

## General Introduction

*Mechanisms of initiation and progression of atherosclerosis*

Atherosclerosis is responsible for nearly 50% of the mortality rate in Europe, the United States, and Japan, and is now understood to be a disease characterized by inflammation that results in a host of complications, including ischemia, stroke, and acute coronary syndromes (unstable angina pectoris and myocardial infarction). A critical event in the early stages of atherosclerosis is focal accumulation of lipid-laden foam cells derived from monocytes/macrophages (Fig. 1). The elevation of plasma atherogenic lipoprotein (low-density lipoprotein; LDL) may lead to its deposition in the intima, where it is rapidly converted to oxidized-LDL (ox-LDL) under pathological conditions. Atherogenic lipoproteins may induce a series of biological changes, including increased numbers of adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), P-selectin, and E-selectin, in endothelial cells, as well as the adhesion and migration of monocytes and T lymphocytes.[1-3] After infiltrating subendothelial sites, monocytes differentiate into macrophages and express

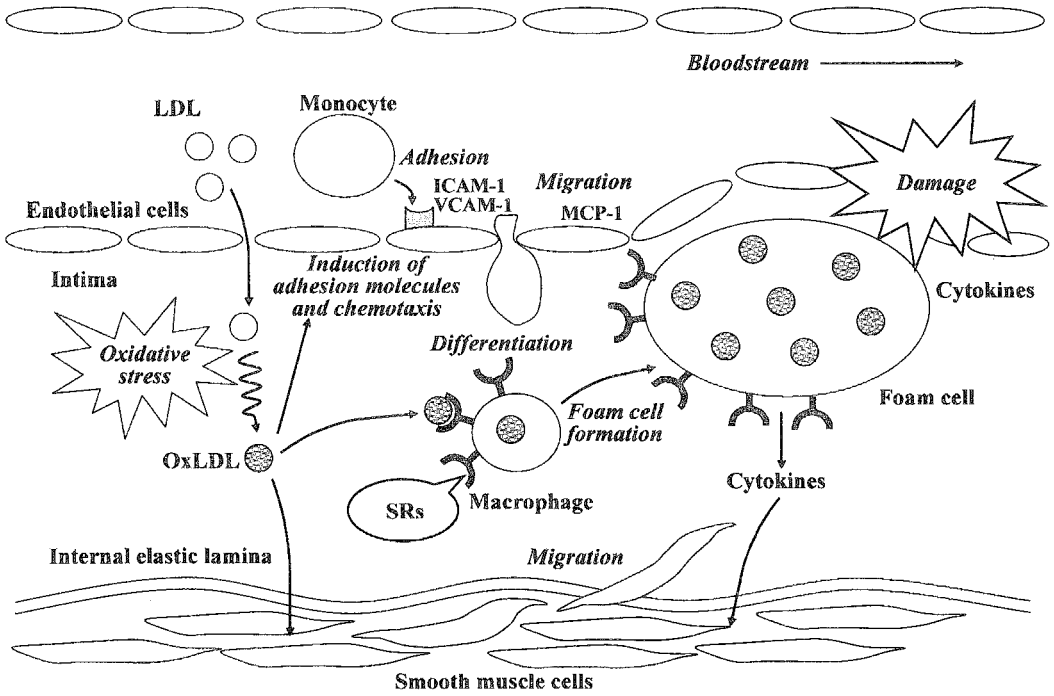


**Fig. 1** Postulated mechanisms of the pathogensis of atherosclerosis. ICAM, intercellular adhesion molecule; LDL, low-density lipoprotein; MCP, monocyte chemoattractant protein; OxLDL, oxidized LDL; SR, scavenger receptor.

several distinct scavenger receptors (SRs), which mediate the endocytic uptake of ox-LDL. Under normal circumstances, uptake of LDL cholesterol via the native LDL receptor (LDLR) is down-regulated with increasing intracellular cholesterol content and internalization of cholesterol by this route does not result in foam cell formation. However, because of the lack of a feedback inhibition mechanism for ox-LDL incorporation via SRs, macrophages are excessively and irreversibly loaded with ox-LDL, and then converted into foam cells.[4-6)]

A large number of proinflammatory cytokines have been shown to be expressed in atherosclerotic lesions, particularly in association with infiltrating monocytes.[7)] Further, soon after a lesion is initiated, there is fragmentation of the internal elastic membrane and migration of smooth muscle cells up into the intima. After migration, these smooth muscle cells express SRs and incorporate ox-LDL, then become foam cells (Fig. 1). An important point is that fatty streak lesions are precursors of more complex lesions that cause inflammation and stenosis, and limit blood flow. These complex lesions ultimately represent the sites of thrombosis leading to myocardial infarction.

*The nature of ox-LDL*

There is abundant evidence that ox-LDL is a key factor in the initiation and progression of the pathology of atherosclerosis.[4,8,9)] Ox-LDL has diverse biological effects, including the expression of pro-inflammatory cytokines and adhesion molecules in arterial wall cells for accelerating atherogenesis,[10)] while it is a chemoattractant for monocytes[11)] and cytotoxic toward endothelial cells in culture,[12)] and can stimulate the release of monocyte chemoattractant protein-1 (MCP-1) and macrophage colony-stimulating factor (M-CSF) from endothelial

**Table 1** Possible proatherogenic effects of oxLDL

| Effects | Possible mechanisms |
|---|---|
| Adhesion of monocytes to endothelial cells ↑ | Increased expression of adhesion molecules on endothelial cells |
| Monocyte and T lympohocyte chemotaxis ↑ | Induction of MCP-1 production and direct chemoattractant effect |
| Scavenger receptors ↑ | Activation of AP-1 and ets transcription |
| Foam cell formation ↑ | Enhanced uptake of ox-LDL mediated by scavenger receptors |
| Induction of proinflammatory genes | Activation of NF-κB, and AP-1, and increased cAMP |
| Increased cellular death | Activation of apoptosis and formation of cholesterol crystals |
| Thrombosis ↑ | Induction of tissue factor, increased platelet aggregation |
| Impaired vascular functions | Dysfunction of endothelin-1 and NO |
| Plaque rupture ↑ | Increased MMPs production |

↑ : enhanced

AP, activator protein; cAMP, cyclic adenosine monophosphate; MMP, matrix metalloproteinase; NF-κB, nuclear factor-kappaB.

cells.[13,14] Moreover, ox-LDL, lysophosphatidyl-choline, and oxidized fatty acids induce the expression of scavenger receptors, such as CD36, SR-A, and LOX-1 (Table 1).

LDL deposits accumulate in vascular tissues under pathological conditions and are rapidly converted to ox-LDL via nucleophilic attack by reactive molecular species, including superoxide ($O_2^-$), hydrogen peroxide and hydroxyl radicals (Fig. 2). These reactive species are generated by endothelium, smooth muscle tissue, and migratory lymphocytes.[15] LDL modification involves changes to both protein and non-protein moieties on the LDL particle.[8,16] Central to the oxidation of LDL is a lipid peroxidation chain reaction that is initiated and driven by free radicals.[17] In this process, lipid hydroperoxides are formed that subsequently fragment to reactive aldehydes, such as malondialdehyde and 4-hydroxynonenal. These can then conjugate to the ε-amino groups of apoB-100 lysine residues and amino phospholipids, such as phosphatidylethanolamine and phosphatidylserine.[18] ApoB-100 also undergoes extensive breakdown during LDL modification, which is due to non-enzymatic oxidative cleavage, with histidine, lysine, and proline residues particularly susceptible to oxidative damage.[19]

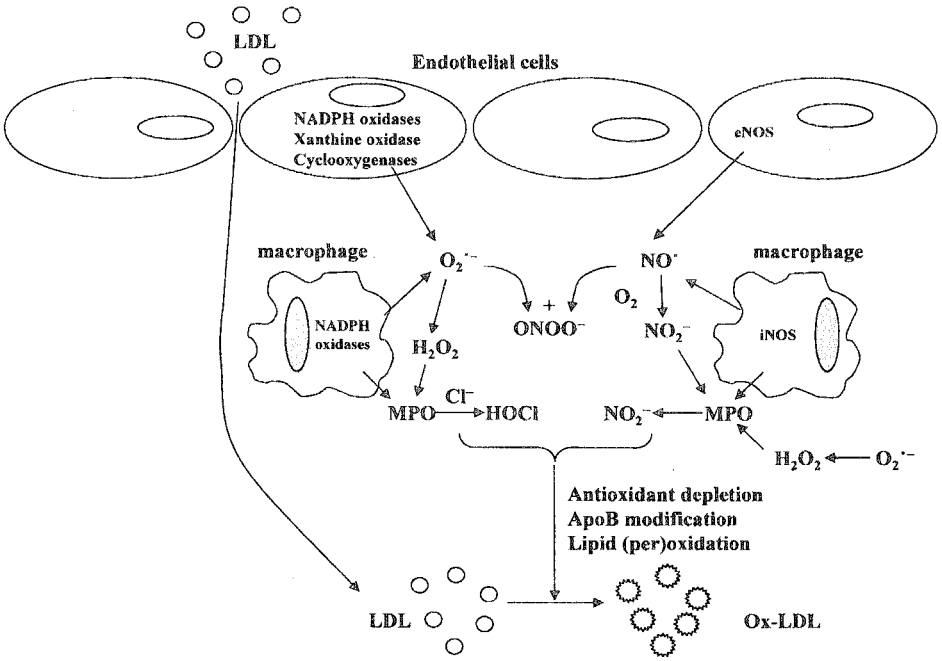


**Fig. 2** A variety of enzymes contribute to the oxidative modification of LDL. iNOS, inducible nitric oxide synthase; MPO, myeloperoxidase; NO, nitric oxide. Reactive oxygen species (ROS): $O_2^{\cdot -}$, superoxide; $ONOO^-$, peroxynitrite; HOCl, hypochlorite; $NO_2^{\cdot -}$, nitrogen dioxide radical. (Modified Ref 53.)

*Scavenger receptors*

The SR family is composed of integral membrane proteins and aptly named, because these receptors have been found to bind and 'scavenge' a broad array of other modified self and nonself ligands, including apoptotic cells, anionic phospholipids, and amyloid and pathogen components.[20] SRs were first described by Brown and Goldstein, and shown to bind and internalized modified forms of LDL through mechanisms not inhibited by cellular cholesterol content,[21] indicating that these receptors are likely participants in macrophage cholesterol accumulation. Since the cloning of the first macrophage SR in 1990,[22] the SR family has expanded to include 8 different subclasses (A-H) of structurally unrelated receptors that share the defining feature of being able to bind modified forms of LDL (Fig. 3, Table 2). [22-34]

Thus far, SR-A (class A SR), CD36 and LIMPII analogous-1 (CLA-1), the human ortholog of SR-BI and CD36 (class B SR), CD68 (class D SR), lectin-like oxidized LDL receptor-1 (LOX-1, class E SR), and the SR

**Table 2** Scavenger receptor family

| Class | SR family member | Ref. No. |
|---|---|---|
| A | SR-AI (class A scavenger receptor I) | 22 |
| | SR-AII (class A scavenger receptor II) | 23 |
| | SR-AIII (class A scavenger receptor III) | 24 |
| | MARCO (macrophage receptor with collagenous structure) | 25 |
| B | SR-BI, II (class B scavenger receptor I, II) | 26 |
| | CD36 (cluster of differentiation 36) | 27 |
| | CLA-1 (CD36 and LIMP-II analogous-I) | 28 |
| C | dSR-CI (drosophila SR-C) | 29 |
| D | CD68 (cluster of differentiation 68) / Macrosialin | 30 |
| E | LOX-1 (lectin-like oxidized LDL receptor) | 31 |
| F | SREC (scavenger receptor expressed by endothelial cells) | 32 |
| G | SR-PSOX (scavenger receptor for phosphatidylserine and oxidized lipoprotein) / CXCL16 (CXC-chemokine ligand 16) | 33 |
| H | FEEL-1,2 (Fasciclin, epidermal growth factor (EGF)-like, laminin-type EGF-like domain-containing scavenger receptor-1,2) | 34 |

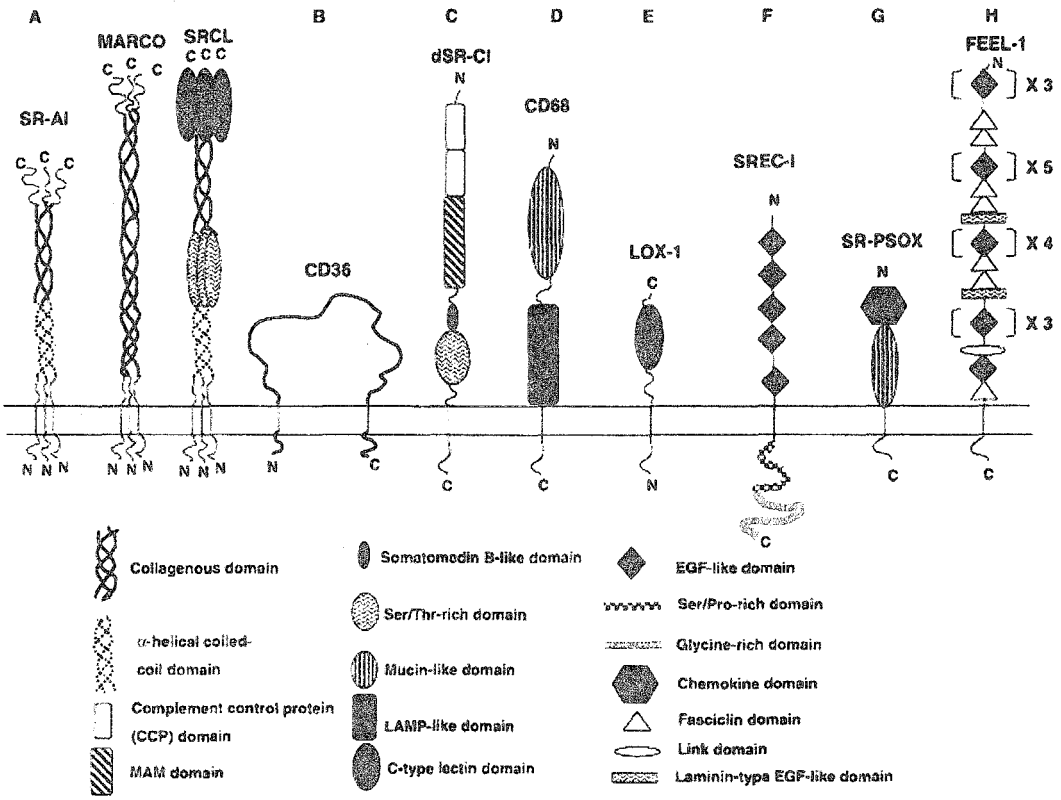


**Fig. 3** Schematic view of different classes of eukaryote scavenger receptors.[23] The eight different classes are denoted A-H and specific domains are highlighted by the codes indicated within the figure. The class C receptor (dSR-CI) is only found in the fruit fly at present. All the other receptor classes have mammalian orthologs. CD, cluster of differentiation; EGF, epidermal growth factor; FEEL, fasciclin, EGF-like, laminin-type EGF-like domain-containing scavenger receptor; LAMP, lysosome-associated membrane glycoprotein; LOX, lectin-like ox-LDL receptor; MAM, meprin A-5 protein and receptor protein tyrosine phosphatase mu; MARCO, macrophage receptor with collagenous structure; SR, scavenger receptor; SRCL, scavenger receptor with C-type lectin domain; SREC, SR expressed by endothelial cells; SR-PSOX, SR for phosphatidylserine and ox-LDL.

for phosphatidylserine and ox-LDL (SR-PSOX or CXCL16, class G SR), have been shown to be responsible for cellular ox-LDL uptake.[20,35] It is important to note that ox-LDL increases the expression of those SRs, resulting in the enhancement of foam cell formation. The expression of SRs is induced by tumor necrosis factor-α (TNF-α), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and other factors,[36,37] which suggests that the *in vivo* expression of SRs may be dynamically regulated by inflammatory and fluid mechanical stimuli.

*SR knockout and transgenic mice*

Pro-atherogenic roles for SRs have been implicated from results of experiments with transgenic and knockout mice. For example, SR-A knockout

mice showed a significant decrease in atherosclerotic lesions as compared to apoE knockout ($apoE^{-/-}$)[38] and LDLR knockout ($LDLR^{-/-}$) mice.[39] Mice susceptible to diet-induced atherosclerosis and lacking SR-A also had significantly reduced atherosclerotic lesion size.[40] In addition, $CD36^{-/-}$ mice crossed with $apoE^{-/-}$ mice and given a high fat diet showed a 70% reduction in atherosclerotic lesion size.[41] Further, experiments with mice lacking both SR-A and CD36 revealed that CD36 is a major ox-LDL receptor required for the formation of foam cells.[42] Transplantation of $CD36^{-/-}$ bone marrow into $apoE^{-/-}$ mice resulted in a large reduction in aortic en face lesion areas in hypercholesterolemic mice, indicating that the macrophage CD36 contributes to lesion progression in the aortic tree.[43] Recently, endothelial overexpression of LOX-1 in $apoE^{-/-}$ mice was shown to enhance ox-LDL uptake and accelerate intramyocardial vasculophathology.[44] These findings suggest that the deletion or repression of SRs may predictably decrease ox-LDL, thereby reducing pathophysiological lesion formation, though a contradictory finding was recently presented by Moore *et al.*, who found that $apoE^{-/-}$ mice lacking SR-A or CD36 showed significant reductions in peritoneal macrophage lipid accumulation *in vivo*, however, increased aortic sinus lesion area, in contrast to previous reports.[45] The conflicting outcomes of these multiple studies on SR involvement in atherosclerosis are difficult to reconcile. Differences among genomic background, hypercholesterolemic mouse models, and diets used in the various studies are likely to have played a significant role in these divergent outcomes, and further studies will be needed to clarify this issue. Further, additional clarification of the roles of LOX-1, SR-PSOX, and other receptors in atherosclerosis is anticipated from future studies of these receptor deficient mice.

In contrast, SR-BI and BII play roles in lipid metabolism by mediating cholesterol uptake from bound high-density lipoprotein (HDL), as well as ox-LDL. SR-BI is postulated to be an anti-atherogenic factor, since SR-BI knockout mice have increased circulating plasma cholesterol levels via HDL.[46] In addition, mice carrying a double knockout of both SR-BI and apoE genes displayed hypercholesterolemia.[47] Further, the atherosclerotic lesion area was found to be increased in SR-BI and apoE deficient mice *in vivo* without influencing plasma lipids, HDL, or cholesterol efflux.[48]

Together, suppression of several SRs may be effective to prevent the development of atherosclerosis.

*Suppression of SRs by synthetic and natural compounds*

In previous studies, expressions of SRs were decreased by natural and synthetic peroxisome proliferator-activated receptor-gamma (PPARγ) ligands.[49,50] In addition, an adipocyte-derived plasma protein, adiponectin, suppressed SR-A expression and subsequent lipid accumulation in human monocyte-derived macrophages.[51] Further, statins, which inhibit the key enzyme in cholesterol biosynthesis, have also been reported to down-regulate those SRs,[52,53] while aspirin was shown to modulate ox-LDL-mediated responses in endothelial cells, including inhibition of reactive oxygen species (ROS) generation and LOX-1 expression.[54] Also, macrophages collected from mice given fish oil had a low level of expression of SR-A,[55] while other studies showed that α-tocopherol decreased the expression of CD36 in smooth muscle cells[56] and monocyte-derived macrophages.[57] Along a similar line, α- and β-tocopherol down-regulated SR-A activity in rabbit peritoneal macrophages.[58] Ide *et al.* indicated that aged garlic extract inhibited homocysteine-induced CD36 expression and foam cell formation in THP-1 cells, a human acute monocytic leukemia cell line.[59] These effects may occur through alterations in signal transduction or through a more direct effect on transcription factors. For example, α-tocopherol down-regulated the DNA binding activity of activator protein-1 (AP-1).[58] Further, the PPARγ ligand pioglitazone reduced intracellular superoxide radical generation, and subsequently the expression of transcription factors NF-κB and AP-1.[50]

*Okinawa longevity*

The residents of Okinawa prefecture are known for having the greatest longevity in the world, according to reports from the World Health Organization (WHO), and the Japan Ministry of Health, Labor and Welfare. Further, cardiovascular disease, including coronary heart disease and stroke, as well as cancer, which are the leading causes of death in many countries, have a very low frequency in Okinawa.[60-62] Other studies have shown that Okinawan people have favorable serum lipid profiles,[63-65] reasonable blood pressure,[65] and the lowest levels of plasma homocysteine,[65,66] which are risk factors of cardiovascular disease in adults. In addition to the comfortable weather conditions and other healthy lifestyle factors including regular physical activity (farming and traditional dance), the unique traditional food habits of Okinawa have been proposed to contribute to the low cardiovascular mortality and long life

expectancy.[65,67] Protective factors may include high anti-oxidant consumption, mainly flavonoids and carotenoids, through high levels of vegetable and soy intake. However, there is limited scientific evidence regarding the physiological roles of those foods and their active components. Of interest, Murakami *et al.* recently found that the anti-oxidative and anti-nitrosative activities of Okinawan food items were remarkably higher *in vitro* than of foods common to the main islands of Japan.[68]

*Evaluation of physiological activities based on bioavailability and metabolism*

In order to more accurately determine the *in vitro* physiological activities of food items and their components, their bioavailability and metabolism in the gastrointestinal tract must be considered, because most plant secondary metabolites are scarcely absorbed and metabolized or chemically decomposed there.[69] Moreover, individual nutrients and interactions with other nutrients must be taken into account. However, only a limited number of *in vitro* experimental methods for precisely investigating small intestinal absorption and gut wall extraction have been conducted. Further, most studies of the absorption of food phytochemicals in humans and experimental animals have required long periods of time for the experiments and a large number of samples. Although some models have been used to determine the intestinal uptake of food components, as well as investigate absorption and intestinal and presystemic metabolism,[70,71] they are complex, time-consuming, and expensive, and occasionally require sophisticated equipment.

The Caco-2 cell line, derived from human colon adenocarcinomas, exhibits enterocyte-like characteristics, such as the expression of brush-border enzymes,[72,73] nutrient transporters,[74,75] and an intercellular tight junction,[73,76] and has been used widely as an *in vitro* model for studies of the small intestine, as well as for studying drug metabolism and transport.[77,78] In addition, many researchers have recently utilized these cells to investigate the absorption and metabolism of dietary carotenoids (β-carotene, lutein, lycopene and fucoxanthin),[79-81] polyphenols (quercetin, apigenin, genistein, daidzein, epicatechin and resveratrol),[75,82-86] and chlorophyll,[80] as well as others.[87,88]

*Objectives and outlines of the present study*

In order to precisely evaluate the *in vitro* physiological functions of plant secondary metabolites, bioavailability and metabolism must be taken into

account. In this context, the author established a novel assay system by focusing on the bioconversion of food constituents using differentiated Caco-2 cells as a model of human intestinal epithelial cells (Chapter I).

Pro-atherogenic roles for SRs have been implicated in results of experiments with knockout mice.[38-42] Those findings suggest that deletion or repression of SRs may predictably decrease ox-LDL uptake, thereby reducing pathophysiological lesion formation. However, there are only a few studies regarding the suppressive effects of food factors on SR expression.[55-58] According to reports from WHO and the Japan Ministry of Health, Labor and Welfare, the residents of Okinawa prefecture have a long life span and very low frequency of cardiovascular disease, compared to other parts of the world. One of the reasons for the longevity of Okinawan people may be their unique traditional food habits. In Chapter II, using the differentiated Caco-2 cell model established in Chapter I, the author evaluated the suppressive effects of 16 traditional food items from Okinawa on TPA-induced LOX-1 mRNA expression in THP-1 human monocyte-like cells. Further, the inhibitory effects of zerumbone, a sesquiterpene from *Zingiber zerumbet* Smith, were investigated.

In Chapter III, the author examined the effects of 5 anti-inflammatory food phytochemicals on TPA-induced SR expression in THP-1 cells. The effect of nobiletin (NOB), a citrus polymethoxylated flavone, on the expression of SRs was subsequently elucidated and the underlying molecular events explored.

While biological activities of NOB have been widely reported, the metabolic fate of NOB has been recently elucidated only in part, and the biological and pharmacological activities of NOB metabolites are yet to be reported. Thus, the suppressive effects of NOB metabolites in rat and mouse urine, as well as a structurally related polymethoxyflavone, tangeretin, on SR expression were evaluated (Chapter IV).

# Chapter I

## Establishment of novel bioassay system for evaluating anti-oxidative activities of food items: Use of basolateral media from differentiated Caco-2 cells

### Introduction

Reactive oxygen and nitrogen species (RONS) are considered to play important roles in the onset of a variety of diseases including atherosclerosis.[89-91] Oxidative modification of LDL is a prerequisite for macrophage uptake and cellular accumulation of cholesterol[4] (Fig. 1), while the release of $O_2^-$ from endothelial or smooth muscle cells might be responsible for lipid peroxidation initiation (Fig. 2). Vascular release of $O_2^-$ is sharply increased in atherosclerotic arteries,[92,93] and $O_2^-$ is known to inactivate nitric oxide (NO) in a chemical reaction during which the cytotoxic radical peroxynitrite ($ONOO^-$) is formed.[94] The presence of $ONOO^-$-derived nitrotyrosine has recently been demonstrated in human atherosclerotic lesions.[95] NO has a vaso-relaxant activity, thus it is very important in the homeostatic function of arterial blood pressure. It was recently reported that the binding of ox-LDL to SRs led to a significant increase in the generation of ROS and in particular $O_2^-$ in endothelial cells, while it also reduced the intracellular concentration of NO.[96] $O_2^-$ and NO are precursors of several types of RONS,[97] and it is widely recognized that suppression of excessive $O_2^-$ and NO generation is an effective and promising method both prevention and therapy for oxidative stress-related diseases. Murakami *et al.* previously reported the inhibitory properties of several food phytochemicals toward $O_2^-$ generation in TPA-activated dimethylsulfoxide (DMSO)-differentiated human promyelocytic HL-60 cells and NO generation in lipopolysaccharide (LPS)/interferon (IFN)-γ-activated RAW 264.7 macrophages.[98-102] Further, some compounds found to be active in *in vitro* experiments have also shown remarkable cancer chemopreventive activities in rodent models, as well as anticipated anti-oxidative properties.[98,102-105]

In this Chapter, the author aimed to establish a novel assay system that reflected *in vivo* anti-oxidative activity by focusing on the bioconversion of food constituents using differentiated Caco-2 cells. Selected food preparations were

added to Caco-2 monolayers on the apical side, and then the inhibitory effects of conditioned media taken from the basolateral side toward $O_2^-$ and NO generation by differentiated HL-60 cells and RAW 264.7 cells, respectively, were measured (Fig. I-1). In addition, transepithelial electrical resistance (TEER) values of the Caco-2 monolayers were determined to examine the effects of the food preparations on the tight junction.

## Materials and Methods

### *Chemicals*

Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS), and IFN-γ were purchased from Gibco BRL (Grand Island, NY). LPS (*Escherichia coli* serotype 0127, B8) was obtained from Difco Labs (Detroit, MI). Cytochrome *c* was from Sigma Chemical (St Louis, MO). All other chemicals were purchased from Wako Pure Chemicals (Osaka, Japan), unless otherwise specified.

### *Cell cultures*

Caco-2 cells, HL-60 human promyelocytic leukemia cells, and RAW 264.7 mouse macrophages were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The Caco-2 and RAW 264.7 cells were maintained in DMEM, and the HL-60 cells in RPMI 1640. Each medium contained 300 ng/mL of L-glutamine supplemented with 10% FBS, 100 U/mL of penicillin, and 100 ng/mL of streptomycin. The cells were incubated under a humidified atmosphere of 95% $O_2$ and 5% $CO_2$ at 37°C, and maintained at 80% to 90% confluence.

For each experiment, Caco-2 cells were seeded on cell culture inserts (PET track-etched membrane, 23 mm in diameter, pore size 0.4 μm, Becton Dickinson Labware, Franklin Lakes, NJ) in 6-well plates at a density of $2\times10^5$ cells/insert. The basolateral and apical compartments contained 2.0 and 3.0 mL of culture medium, respectively. Medium was replaced freshly 2 or 3 times a week. The cell culture inserts were used for the experiments 14–21 days after seeding, when TEER exceeded 300 $\Omega cm^2$.

### *Measurement of TEER*

The TEER value of the Caco-2 monolayers was measured according to

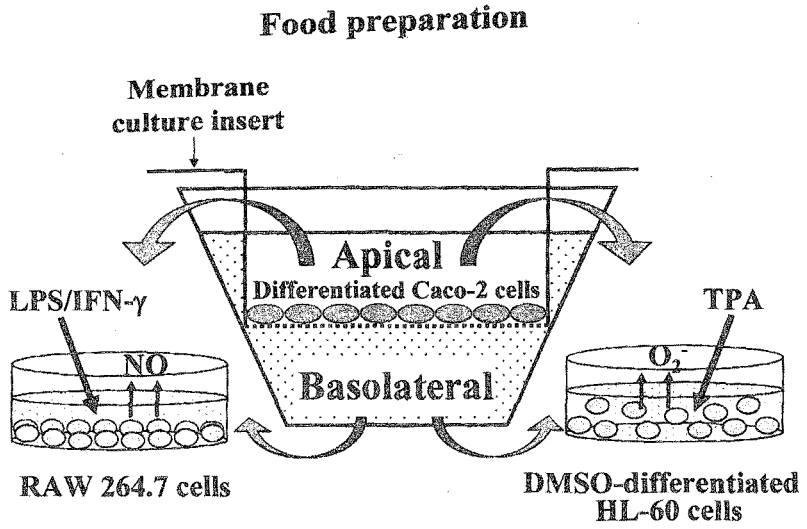


**Fig. I-1** Illustrative outline of the present novel anti-oxidative assay system using differentiated Caco-2 cell monolayers. Caco-2 cells were seeded on cell culture inserts in 6-well plates at a density of $2\times10^5$ cells/insert and fully differentiated. Each fresh food preparation was added to the apical side of the monolayer at various final concentrations and incubated at 37°C for the designated times. After incubation, the TEER values of the Caco-2 monolayers were measured. Each conditioned medium and food preparation were separately tested for their inhibitory effects on TPA-induced $O_2^-$ generation in differentiated HL-60 cells and on combined LPS/IFN-γ-induced NO generation in RAW 264.7 macrophages. The viability of Caco-2, RAW 264.7, and HL-60 cells was determined by MTT and Trypan Blue dye exclusion tests, respectively.

the method of Hidalgo *et al.* using a Millicell-ERS instrument (Millipore Co., Bedford, MA).[73] The measurements were performed prior to and at the end of each experiment to assess the integrity of the Caco-2 monolayers. Monolayer resistance, obtained by subtracting intrinsic resistance (membrane alone) from total resistance (membrane plus monolayer), was corrected for the surface area and expressed as $\Omega cm^2$. The effect of each assay sample on TEER was expressed as the relative value, obtained by correcting the control value [phosphate-buffered saline (PBS)-treated values as 100%].

*Sample preparation*

Fresh food items [ginger, garlic, *shimeji* (*Hypsizigus marmoreus*, a mushroom), onions, and carrots] were purchased from a local supermarket in Kyoto, Japan. Ten grams of each were cut into small pieces, suspended in 10 mL of PBS, and subjected to a homogenizer (Ultra Turrax T25 basic, IKA Labortechnik, Staufen, Germany) for 30 sec. at room temperature. Then, the homogenates were centrifuged at 5000 × *g* for 5 min and filtrated through filter paper (P3801 No. 2 Advantec Tokyo Japan). Each resulting food preparation was

aliquoted and frozen immediately at –80°C until use.

*Food preparation-treated media from Caco-2 monolayer*

Fully differentiated Caco-2 cells, prepared as described above, were used for the experiments. The medium from each side was removed and the inserts were washed with HBSS twice. Phenol-red free DMEM medium was then added to the apical (2 mL) and basolateral (3 mL) sides. Then, one of the food preparations or PBS at various volumes (0–25%, *v/v*) was added to the apical side of the Caco-2 monolayers and incubated at 37°C for the designated time periods. After incubation, the medium from both sides was independently collected and the anti-oxidative activity of the each resultant medium preparation was evaluated as described below. The viability of the Caco-2 cells was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[106)]

*TPA-induced $O_2^-$ generation in differentiated HL-60 cells*

An inhibitory test of TPA-induced $O_2^-$ generation was performed as previously reported with some modifications.[102)] HL-60 cells were pre-incubated with 1.25% DMSO in RPMI 1640 medium for 6 days and differentiated into granulocytes. Differentiated HL-60 cells ($1\times10^6$) were incubated in 1 mL of each food preparation, or apical or basolateral medium at 37°C for 15 min. Following the pre-incubation, the cell suspension was centrifuged and washed with HBSS twice, then suspended in 1 mL of HBSS. Ninety seconds after stimulation with 100 nM of TPA, cytochrome *c* (1 mg/mL) was added to the reaction mixture and incubated for 15 min at 37°C. Visible absorption by the supernatant at 550 nm, due to reduced cytochrome *c*, was measured for $O_2^-$ generation. The viability of HL-60 cells was determined using a Trypan Blue dye exclusion test. Cells treated with only the vehicle and those with TPA alone were used as negative and positive controls, respectively.

*LPS/IFN-γ-induced NO generation in RAW 264.7 cells*

Inhibitory tests of LPS/IFN-γ-induced NO generation were performed as previously reported with some modifications.[101)] RAW 264.7 mouse macrophages ($2\times10^5$ cells/mL) in 1 mL of DMEM medium on a 24-well plate were pre-incubated at 37°C for 18 h. After the cells were washed with PBS twice, 1 mL of each food preparation, or medium from the apical or basolateral side

incubated with each food preparation, was added to the cells. Following incubation for 30 min, LPS (100 ng/mL), IFN-γ (100 units/mL), and L-arginine (2 mM) were added to the medium. After 24 h, the levels of nitrite ($NO_2^-$) and cytotoxicity were measured using Griess[107] and MTT assays, respectively. Cells treated with only the vehicle and with LPS/IFN-γ alone were used as negative and positive controls, respectively.

*Statistical analysis and inhibitory rate (IR)*

Each experiment was done independently at least 3 times and the data are expressed as the mean ± standard deviation (SD). The statistical significance of differences between groups in each assay was assessed by a Student's *t*-test (two-sided) that assumed unequal variance. The IR in each assay was calculated using the following equation: IR (%) = {1 − [(test sample data) − (negative control data)][(positive control data) − (negative control data)]$^{-1}$} × 100.

## Results

*Suppressive effects of food preparations, and conditioned apical and basolateral media on $O_2^-$ generation*

The ginger, garlic, *shimeji*, onion, and carrot preparations were selected as test samples, based on the results of a previous screening test,[108] after being made from a homogenate at a concentration of 1 g/mL of fresh PBS followed by filtration. Each preparation at a concentration of 25% (*v/v*) showed 90% or greater inhibition toward TPA-induced $O_2^-$ generation in differentiated HL-60 cells (Fig. I-2A). Further, the garlic [cell viability (CV) = 0%] and *shimeji* (CV < 20%) preparations showed substantial cytotoxicity toward HL-60 cells. Next, each preparation was added to the apical side of the Caco-2 monolayers at a concentration of 25% (*v/v*), and the apical and basolateral media were separately collected after 24 h of incubation. The apical medium samples treated with garlic, *shimeji*, and carrot preparations showed significant inhibitory effects [inhibitory rate (IR) > 80%] (Fig. I-2B), while the ginger- and onion-conditioned apical media (IR < 20% and < 40%, respectively) were less active. In addition, no notable cytotoxicity was observed (CV > 80%) for any medium sample from the apical side of Caco-2 cells treated with the preparations for 24 h (Fig. I-2B). It was notable that only the garlic-conditioned basolateral media abolished $O_2^-$ generation without any marked cytotoxicity toward HL-60 cells (IR = 100, CV >

85%) (Fig. I-2C). Both the onion and carrot-conditioned basolateral medium showed moderate inhibition (IR ~ 40 %), and that treated with ginger or *shimeji* had weaker activity (IR ~ 20 %).

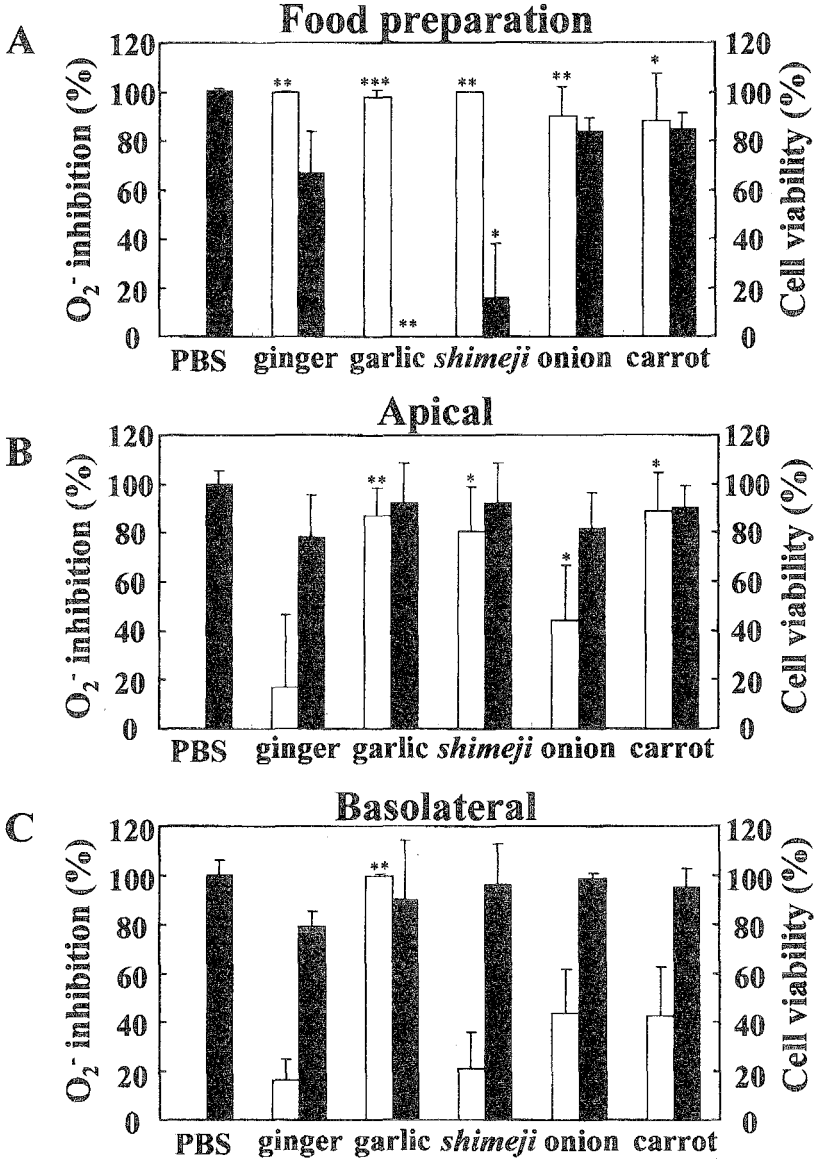


Fig. I-2 Suppressive effects of food preparations (A), and apical (B) and basolateral (C) medium samples on TPA-induced $O_2^-$ generation in differentiated HL-60 cells. Open bars; inhibitory rates, closed bars; HL-60 cell viability. HL-60 cells were pre-incubated with 1.25% DMSO in RPMI 1640 medium for 6 days and allowed to differentiate into granulocytes. Differentiated HL-60 cells ($1\times10^6$) were incubated with 1 mL of each food preparation (25% *v/v*), or apical or basolateral medium samples for 15 minutes at 37°C. Following that pre-incubation, the suspension was centrifuged and washed with HBSS twice, then the cells were suspended in 1 mL of HBSS. Ninety seconds after stimulation with TPA (100 nM), cytochrome C (1 mg/mL) was added to the reaction mixture and incubated for 15 minutes at 37°C. Visible absorption by the supernatant was measured at 550 nm to determine $O_2^-$ generation. HL-60 cell viability was determined by a Trypan Blue dye exclusion test. Values are shown as the means ± SD (n = 3). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus equivalent volume of PBS-treated control using Student's *t*-test.

*Suppressive effects of food preparations, and conditioned apical and basolateral media on NO generation*

Each preparation (25% *v/v*)-conditioned basolateral medium suppressed LPS/IFN-γ-induced NO generation in RAW 264.7 cells by 90% or more (data not shown), therefore, they were retested at a concentration of 5% (*v/v*). Although all

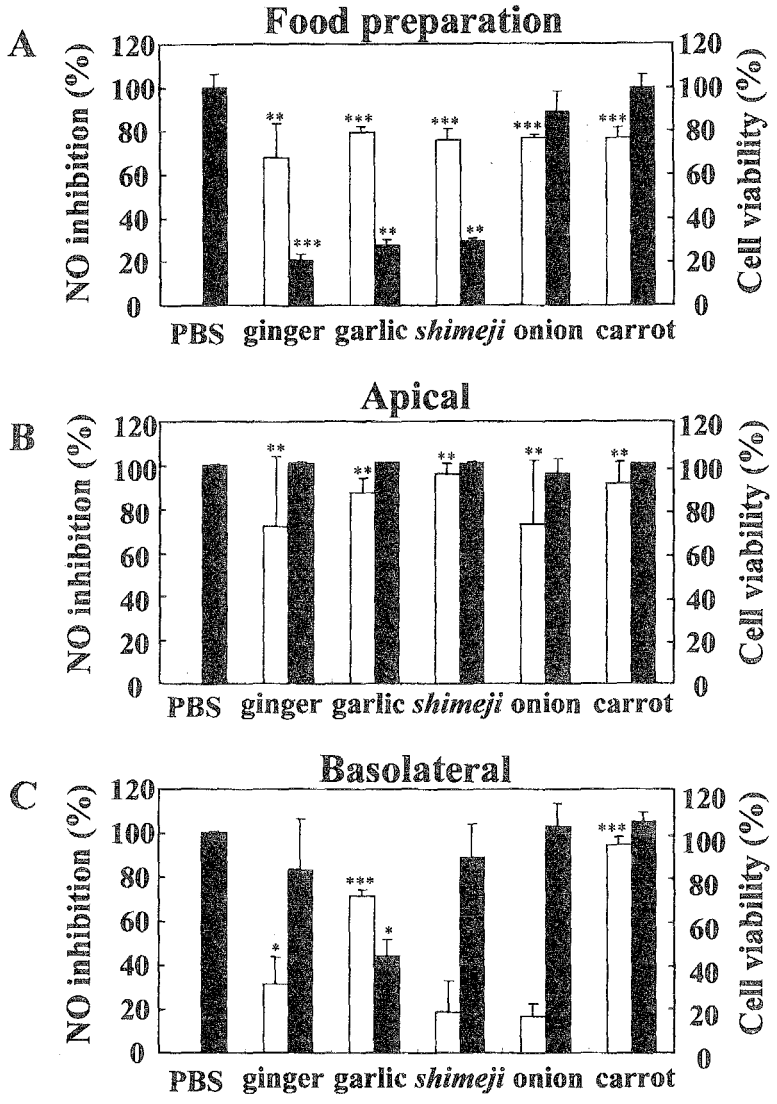


**Fig. I-3** Inhibitory effects of food preparations, and apical and basolateral medium samples on LPS/IFN-γ-induced NO generation in RAW 264.7 cells. Open bars; inhibitory rates, closed bars; cell viability. RAW 264.7 cells ($2\times10^5$) were incubated in 1 mL of the food preparations (25% *v/v*) (A), or apical (B) or basolateral (C) medium samples for 30 minutes at 37°C. Following the pre-incubation, the cells were treated with LPS (100 ng/ml), IFN-γ (100 units/ml), and L-arginine (2 mM) for 24 hours at 37°C. After 24 hours, the levels of $NO_2^-$ were measured using a Griess assay. Cell viability was determined using MTT assays. Values are shown as the means ± S.D. (n = 3). $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$ versus equivalent volume of PBS-treated control using Student's *t*-test.

of the preparations (5% *v/v*) highly suppressed NO generation (IR > 70%), the ginger, garlic, and *shimeji* preparations were cytotoxic (CV < 30%) (Fig. I-3A). Further, it is interesting that apical medium collected from Caco-2 cells treated separately with those 3 preparations were not cytotoxic (CV > 95%) (Fig. I-3B). In addition, all 5 food preparations maintained their potent NO suppressive effects (IR > 70%) when exposed to the Caco-2 monolayers for 24 h (Fig. I-3B). As shown in Fig. I-3C, the ginger- (IR < 40%), *shimeji*- (IR < 20%) and onion- (IR < 20%) conditioned basolateral media showed weak suppression, while the garlic-conditioned medium was cytotoxic (IR = 71.2%, CV < 50%) toward RAW 264.7 cells. It should be also be noted that the carrot-conditioned basolateral medium exhibited pronounced suppression without any cytotoxicity (IR = 90%, CV = 100%).

*Effect of food preparations on Caco-2 cell viability and TEER*

The cytotoxicity and TEER values of the Caco-2 cell monolayers were determined to investigate the effects of the tested food preparations on CV and permeability through the tight junction, respectively, of Caco-2 cells. As shown in Fig. I-4A, the values for CV were 90% or more when the cells were exposed to the ginger, *shimeji*, or carrot preparations (25% *v/v*) for 24 h, whereas their relative TEER values decreased significantly by 40% to 50% as compared with

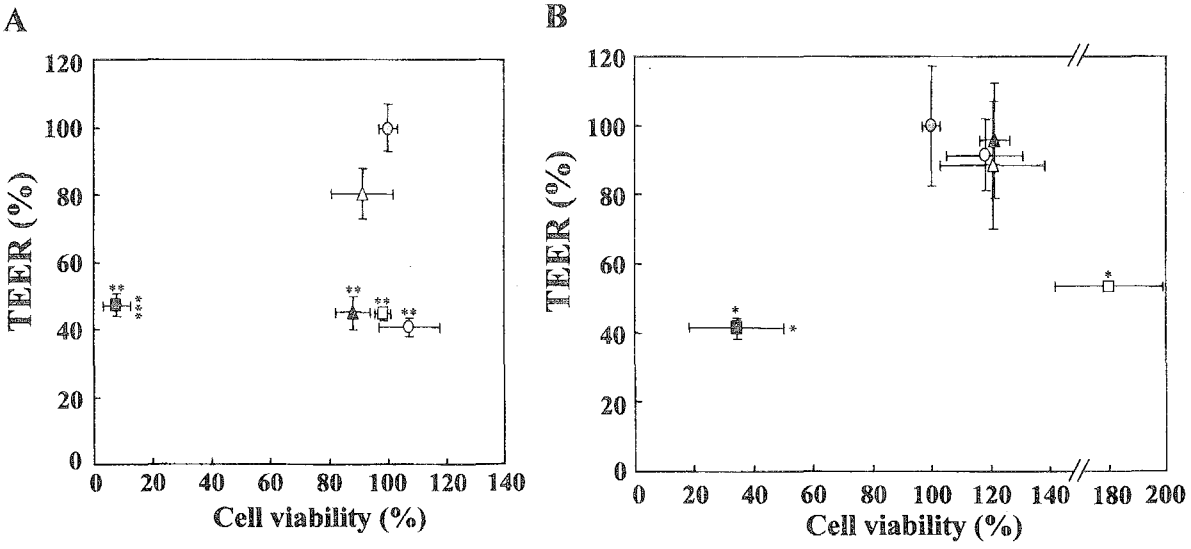


**Fig. I-4** The effects of food preparations on TEER value and Caco-2 cell viability. Caco-2 cells monolayers were incubated with the food preparations at 25% (*v/v*) (A) and 5% (*v/v*) (B) concentrations for 24 hours. The TEER values are presented as relative to the value of the control (PBS). Values are shown as the means ± S.D. (n = 3). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ versus equivalent volume of PBS-treated control using Student's t-test, ●: PBS, ▲: ginger, ■: garlic, ○: *shimeji*, △: onion, □: carrot

the control. On the other hand, a garlic preparation at the same concentration was highly cytotoxic toward Caco-2 cells (10% > CV) and also decreased the relative TEER value by 50%. It is notable that an onion preparation did not significantly affect the values for CV and TEER. When the sample concentrations were reduced to a concentrations of 5% (*v/v*) (Fig. I-4B), the garlic preparation still exhibited substantial cytotoxicity (CV < 35%) and reduced the TEER value by 60%, as compared with the vehicle-added medium. In addition, the carrot preparation decreased the TEER value by 50% and increased CV by 80%. In contrast, the other preparations did not significantly change the CV or TEER values when compared with the control.

*Concentration- and time-dependent suppressive effects of carrot preparation-treated basolateral medium on NO generation*

Since the effects of carrot-conditioned basolateral medium were found to be promising, it was used for further investigations of concentration- and time-dependent activities toward NO suppression. When the Caco-2 cell monolayer was exposed to 0.04% to 1.0 % (*v/v*) of the carrot preparation, the inhibitory effect of the apical medium was remarkable (IR ~ 80%), though it

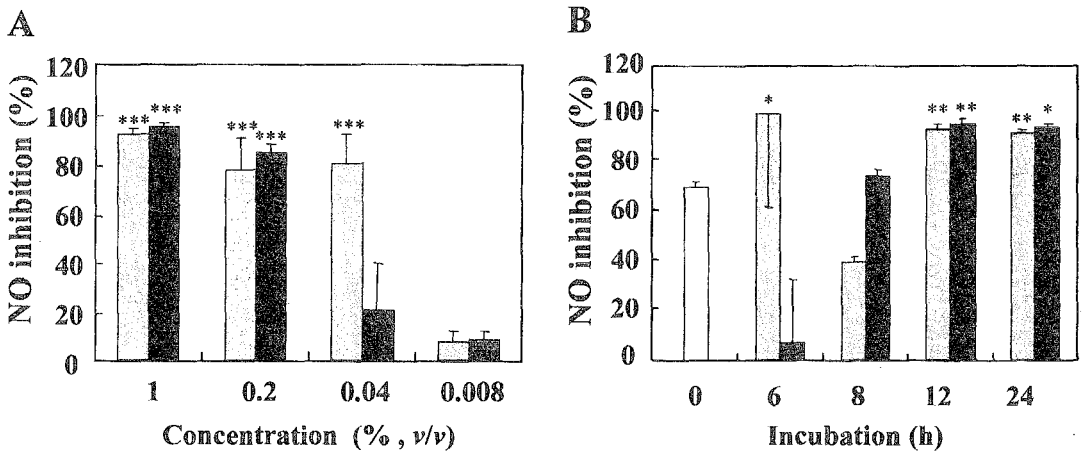


**Fig. I-5** Concentration- and time-dependency of carrot preparation, and apical and basolateral medium treated with the carrot preparation on NO generation in RAW 264.7 cells. (A) Carrot preparations at concentrations ranging from 0.008% to 1.0% (*v/v*) were added to the apical side of the Caco-2 monolayers for 24 hours. After incubation, apical (bias bars) and basolateral (solid bars) media were collected separately, and their inhibitory effects on LPS/IFN-γ-induced NO generation in RAW 264.7 cells were measured using Griess assay. (B) Carrot preparations at a concentration of 1% were added to the apical side of the Caco-2 monolayers for 0, 6, 8, 12, and 24 hours. At each incubation time point, the preparation without the Caco-2 monolayer (open bar), and apical (bias bars) and basolateral (closed bars) media were collected, and their inhibitory effects toward LPS/IFN-γ-induced NO generation in RAW 264.7 cells were measured using a Griess assay. Values are shown as the means ± S.D. (n = 3). $*P < 0.01$, $**P < 0.001$ versus equivalent volume of PBS-treated control using Student's *t*-test.

declined at a concentration of 0.008% (*v*/*v*). On the other hand, the inhibitory effects of the carrot (0.2–1.0%, *v*/*v*)-conditioned basolateral medium were high (IR > 84%), and then became drastically diminished at concentrations of 0.04% or lower (*v*/*v*) (Fig. I-5A). In the time-course results (Fig. I-5B), the value at 0 h indicates the IR of the carrot preparation itself (1%, *v*/*v*) without incubation with Caco-2 cells. There was no time-dependency for the NO generation suppressive effect of the apical medium. In contrast, there was a linear increase in the IR results of carrot (1%, *v*/*v*)-conditioned basolateral medium samples from 6 to 12 h, which was sustained until 24 h. Interestingly, the inhibitory effects of the carrot-conditioned apical and basolateral media (IR > 93%) at 12 and 24 h, respectively, were significantly higher than those of the preparation itself (IR = 70%) (Fig. I-5B).

**Discussion**

Activated leukocytes induce oxidative stress via the activation of NADPH oxidase and inducible nitric oxide synthase (iNOS) to generate $O_2^-$ and NO, respectively. Dietary factors have biological potentials to alternate generation of these free radicals through several modes of actions including: (1) alternation of stimuli-induced activation of signal transduction pathways for NADPH oxidase and iNOS; (2) induction of anti-oxidant enzymes such as SOD and catalase; and (3) scavenging ROS. Since the present experimental systems could only estimate the sum of these effects, the detailed modes of anti-oxidation of active food preparations remain to be clarified.

For the present study, the author selected 5 food items for testing based on previous data showing that their chloroform extracts demonstrated notable suppressive activities toward phorbol ester-induced $O_2^-$ generation in differentiated HL-60 cells.[108] The food preparations were prepared from homogenates, not extracts, which is in contrast to most reported anti-oxidative assay systems that have been used to determine the activities of pure chemicals or samples prepared as alcohol extracts, whereas the chemical compositions of the extracts with water were considered to more closely resemble those of the food preparations used in this study. The author considered that the present experimental approach utilizing food preparations may be more practical, because epithelial cells in the small intestinal tract are exposed to considerably higher concentrations of food digestive compounds after ingestion, the

characteristics of which resemble food preparations more closely than chemicals or alcohol extracts. In addition, a recent study found that the complex mixture of phytochemicals contained in fruits and vegetables provides a more beneficial effect toward health promotion and disease prevention through overlapping or complementary effects than isolated phytochemicals.[109] However, the present *in vitro* assay system did not integrate some of the metabolic and digestive aspects that are important for mimicking *in vivo* situations, as ingested meals are subjected to a two-phase process in humans. Namely, following the gastric phase, which includes acidification and treatment with pepsin, the intestinal phase is initiated by neutralization, then continues with the addition of pancreatin, lipase, and bile acids. It would be desirable to utilize those factors to improve the validity and predictive ability of present assay system. This notion is supported by a report by Ferruzzi *et al.*, who showed that the small intestinal uptake of carotenoids and chlorophyll derivatives from spinach puree was significantly promoted by use of an *in vitro* digestion method with the Caco-2 model.[80,110]

Interestingly, the inhibitory activities of the apical medium samples were decreased, while cell viability increased, after 24 h of incubation, as compared to the corresponding preparations alone. For example, the $O_2^-$ generation inhibitory effects of apical medium from ginger (IR = 100–17%) and onion (IR = 90–44%) were largely decreased (Figs. I-2AB). Additionally, the viability of HL-60 cells was increased when garlic (CV = 0–90%) and *shimeji* (CV = 16–90%) were incubated with the Caco-2 monolayers (Figs. I-2AB). In the NO generation experiment, the cytotoxicity of apical medium disappeared when the cells were treated with ginger (CV = 20–100%), garlic (CV = 27–100%), or *shimeji* (CV = 30–100%) preparations (Figs. I-3AB). These results imply that certain components responsible for anti-oxidative activity and cytotoxicity drastically changed by chemical degradation and/or biological effects of the Caco-2 monolayer. Further, present findings showed that conventional anti-oxidation tests do not have the ability to detect such changes, some of which may occur as a part of metabolic processes in the small intestine. In the present experiment condition, the author could find the inhibitory effect on ROS generation of basolateral medium after incubation with differentiated Caco-2 cells, however, the mechanism point has to be clarified.

The efficiency of intestinal absorption of non-nutrients is controlled by a variety of factors, including gastrointestinal secretion, peristaltic activity in the intestine, the physiological condition of the epithelial membrane, and the

mucosal immune system. Various regions of the gastrointestinal tract are normally exposed to a wide range of diverse molecules and microorganisms. In the present assay system, the author speculated that some components existed in the basolateral medium after incubation with differentiated Caco-2 cells. These might be classified into: (1) food components that penetrate the paracellular pathway or tight junction with an intact structure; (2) food component metabolites, including conjugates with glucuronic and sulfuric acids and/or methylated derivatives; (3) chemically degraded products; and (4) secreted factors, including hormones and cytokines from Caco-2 cells in response to stimulation from the food preparation stimuli; as well as others.

It is important to note that differentiated Caco-2 cells have been reported to express UDP-glucuronosyl-transferase[111,112] and sulfotransferase,[113-115] and that quercetin-4'-glucoside, present in onions, is hydrolyzed by β-glucosidase[82,84,116] or lactase phlorizin hydrolase in this cell line. Further, Caco-2 cells are known to release various cytokines, including interleukin-6 (IL-6) or IL-8, in response to a number of different food factors, such as human milk factors, oleic acid, capric acid, butyrate, and *Bacillus subtilis* present in *natto*, as well as others.[117-120] Kuo *et al.* reported that the level of the metal binding antioxidant protein, metallothionein, increased when genistein and biochanin A were added to Caco-2 cells.[121] These findings may help to identify the active components responsible for the suppression of $O_2^-$ and NO generation, which not be components of the original food.

The paracellular pathway is thought to provide a highly dynamic transport route for certain ions and macromolecules, thereby contributing to the intestinal transport of various nutrients. Food components that are orally ingested and present in the intestinal lumen are also likely to participate in regulating tight junction permeability. TEER, a highly sensitive parameter for membrane permeability, is regulated by membrane-perturbing toxicants and a decrease in TEER is a clear indication of an increase in cell permeability caused by toxins that enhance tight-junction permeability.[122,123] In the present study, only the carrot preparation decreased TEER without cytotoxicity to Caco-2 cells at a lower concentration (Fig. I-4B). Ginger and *shimeji* preparations had a propensity to decrease TEER with increasing concentration of these preparations (Figs. I-4AB). Along a similar line, Xu *et al.* evaluated the effects of lime, lemon, grapefruit, and pummelo juices on a Caco-2 cell line, and found that TEER decreased with increasing concentrations of the lime and lemon juices, while the

grapefruit and pummelo juices increased TEER at high concentrations.[124] Hashimoto *et al.* also reported that a sweet pepper extract enhanced tight junction permeability.[125] Additionally, various food factors, such as chitosan,[126] saponin,[127] and capric acid,[128] as well as others, have shown similar effects, whereas piperine[129] and eicosapentaenoic acid[130] have been demonstrated to have contrasting effects.

One of the intriguing findings of the present study was that the inhibitory activity of the carrot-conditioned basolateral medium toward NO generation after 12 and 24 h was higher than that of the carrot preparation itself at the same concentration (Fig. I-5B). This may indicate that unknown factors responsible for NO suppression were newly produced in the Caco-2 cell monolayer in response to the carrot preparation. The author has not identified the active component present in carrot preparation-conditioned basolateral medium. In addition, the viability of the carrot preparation-treated Caco-2 cells exceeded 180%, as measured by an MTT test (Fig. I-4B), suggesting that the carrot preparation might stimulate the mitochondria in Caco-2 to generate ROS, which are known to reduce MTT.[131] Nevertheless, the viability of the carrot preparation-treated Caco-2 cells should be determined with other systems, including the release of cytosolic lactate dehydrogenase (LDH) and Trypan Blue-dye exclusion tests.

In conclusion, when exposed to Caco-2 cells, 5 different food preparations exhibited a wide range of antioxidant activities, cytotoxicity, and effects on the tight junction, suggesting that the present novel bioassay system may be appropriate to determine the *in vivo* anti-oxidative efficacy of dietary anti-oxidants following ingestion.

## Chapter II

## Zerumbone suppresses phorbol ester-induced expression of multiple scavenger receptor genes in THP-1 human monocytic cell

### Introduction

One of the earliest events in atherosclerosis is accumulation of ox-LDL in the intima, and this modified LDL is a key factor in the initiation and progression of the pathology of atherosclerosis.[4,8,9] SR-mediated recognition of ox-LDL by macrophages leads to formation of foam cells and visible lesions,[4-6,132,133] and pro-atherogenic roles for SRs have been implicated by experiments with knockout mice.[38-42] Those findings suggest that deletion or repression of SRs may predictably decrease ox-LDL uptake, thereby reducing pathophysiological lesion formation.

Macrophages have a central role as effector cells at sites of chronic inflammation, such as those associated with atherosclerotic plaque. The expression of SRs is upregulated during the differentiation of monocytes into macrophages, which is a key event in the process of atherosclerosis. Macrophages excessively uptake ox-LDL through SRs, leading to their conversion into foam cells.[4-6] Further, they are known to be regulated by TNF-α, TPA, and ox-LDL,[36,37] suggesting that SR expression *in vivo* may be dynamically regulated by inflammatory and fluid mechanical stimuli. Several studies have also shown that targeted deletion of either SR-A or CD36 in hyperlipidemic mouse models leads to a reduction in atherosclerotic lesions.[38,39,41] Similarly, Kunjathoor *et al.* reported that macrophages lacking both SR-A and CD36 demonstrated a reduction of 80% to 90%, respectively, in internalized and degraded ox-LDL and acLDL.[42]

In Chapter I, the author established a novel bioassay system to focus on the bioconversion of food constituents using differentiated Caco-2 cells as a model of the small intestine. In this Chapter, the author focused on the Okinawan traditional food items, because the residents of Okinawa are known for having the highest longevity in Japan and a low rate of death due to cardiovascular disease.[60-62] Using the Caco-2 cell model, the author evaluated the suppressive effects of 16 traditional food items from Okinawa on TPA-induced LOX-1 mRNA expression in THP-1 cells and found that the zerumbone, a sesquiterpene,

permeated into the basolateral side of Caco-2 cell monolayers for suppressing the expression of multiple SRs.

## Materials and Methods

*Materials*

Opti-MEM® was purchased from Gibco BRL (Grand Island, NY). Oligonucleotide primers were synthesized by Proligo (Kyoto, Japan). A QIAshredder™ and RNeasy Mini Kit® were purchased from Qiagen (Hilden, Germany), and an RNA PCR Kit (ver. 2.1, AMV) from TaKaRa Bio (Shiga, Japan). pRL-TK (*Renilla* luciferase) and a Dual-Luciferase Reporter Assay System were purchased from Promega (Madison, WI). Lipofectamine was purchased from Invitrogen (Carlsbad, CA). Acetylated LDL labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindo-carbocyanine perchlorate (DiI) was obtained from Biomedical Technologies, Inc. (Stoughton, MA). Zerumbone was purified as previously reported.[134] All other chemicals were the same as in Chapter I, unless specified otherwise.

*Cell culture*

Human monocyte-derived THP-1 cells (ATCC, Manassas, VA) were maintained in RPMI 1640 medium supplemented with 10% FBS, 100 ng/mL of streptomycin, 100 U/mL of penicillin, and 300 ng/mL of L-glutamine. Caco-2 cells were incubated as shown in Chapter I.

*Sample preparation*

Fresh food items were purchased at local markets in Okinawa prefecture, Japan. Ten grams of each were cut into small pieces, suspended in 10 ml of PBS, and subjected to a homogenizer (Ultra Turrax T25 basic, IKA Labortechnik, Staufen, Germany) for 30 sec. at room temperature. The homogenates thus obtained were centrifuged at 5000 × *g* for 5 min and filtrated through a filter paper (P3801 No. 2) (Advantec, Tokyo, Japan). For mimicking *in vivo* situations, each sample was subjected to a two-phase process of the gastric phase, including acidification, and the intestinal phase was initiated by neutralization. Each sample was acidified to pH 2 with HCl, then incubated with shaking at 37°C for 6 h in a water bath. Next, the sample was neutralized to pH 7 with $NaHCO_3$ and incubated at 95°C for 10 min in a heat block. Each resulting food preparation was

aliquoted and frozen immediately at −80°C until use.

*Suppressive effects of basolateral media on TPA-induced LOX-1 mRNA expression in THP-1*

Fully differentiated Caco-2 cells, prepared as described in Chapter I, were used for the experiments.[135] The medium from each side of the insert was removed and it was washed with HBSS twice. Phenol-red free DMEM medium was then added to the apical (2 ml) and basolateral (3 ml) sides. Next, one of the test food preparations (5% or 25%, *v/v*) or PBS was added to the apical side of the Caco-2 monolayer and incubated at 37°C for 24 h. After incubation, $1.5\times10^6$ THP-1 cells were incubated in the medium collected from the basolateral side (1 ml) in a 12-well plate for 30 min, followed by stimulation with TPA (30 nM) for 24 h. The suppressive effects on LOX-1 mRNA expression were evaluated by reverse transcription-polymerase chain reaction (RT-PCR), as described below. The cell viability (CV) of the Caco-2 cells was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay[106] and that of THP-1 cells was determined using a trypan blue-dye exclusion test.

*Suppressive effects of zerumbone and its analogs on TPA-induced SR mRNA expression in THP-1 cells*

THP-1 cells at $1.5\times10^6$ were incubated with the test samples or vehicle (0.5% DMSO, *v/v*) for 30 min in 1 ml of RPMI medium with 10% FBS in 12-well plates, followed by stimulation with TPA (30 nM) for the designated periods of time. The suppressive effect of each test sample on LOX-1 mRNA expression was evaluated by RT-PCR, as described below.

*RT-PCR*

Total RNA was extracted using a QIAshredder™, RNase-free DNase set, and an RNeasy Mini Kit®. A *glyceraldehyde-3-phosphate-dehydrogenase* (*GAPDH*) transcript served as the internal control. cDNA was synthesized using 1 μg of total RNA and an RNA PCR Kit (AMV). PCR amplification was performed using a thermal cycler (PTC-100™, MJ Research, Watertown, MA), and conducted with 0.05 μM of each sense and antisense primer. The primer sequences and PCR conditions (denaturation, annealing, and primer extension time and temperature) are listed in Table II-1. PCR products were subjected to electrophoresis on 3% agarose gels and stained with SYBR® Gold. The signal

intensities of the bands with the expected sizes were quantified by NIH Image and compared with that of *GAPDH* amplified under identical conditions.

*Cellular uptake of DiI-acLDL*

THP-1 cells were cultured on chamber slides (IWAKI, Tokyo, Japan) ($1\times10^5$ cells/0.2 ml RPMI medium with FBS 10%) and incubated for 30 min with zerumbone (10 μM) or the vehicle (0.5% DMSO, *v/v*). After being stimulated with TPA (30 nM), the cells were incubated at 37°C for 48 h then washed 3 times, placed in RPMI medium containing DiI-acLDL (10 μg/ml), and incubated at 37°C for 4 h. The medium was removed and the cells were washed 3 times with PBS, then fixed with 4% paraformaldehyde for 20 min at room temperature, followed by washing 3 times with distilled water. A drop of 80% PBS in glycerol was mounted on the slide glass prior to viewing. DiI-acLDL uptake was observed with UFX-35A fluorescent microscope (Nikon, Tokyo), with the results shown as the original magnification × 200.

*Reporter assay*

Cells were transfected with 4 μg of AP-1 or nuclear factor-kappa B (NF-κB) promoter-luciferase constructs with the herpes simplex thymidine kinase driven Renilla luciferase reporter (pRL-TK) plasmid (Promega) using lipofectamine. They were then incubated at a concentration of $5\times10^5$ cells/ml in Opti-MEM® I medium containing the transfection mixture for 12 h at 37°C. After transfection, the transfection reagent was replaced with RPMI medium containing 10% FBS for 12 h. The cells were next plated in 24-well plates and treated with zerumbone (10 μM) or the vehicle for 30 min then stimulated with TPA (30 nM) for 48 h. pRL-TK was used in the co-transfection experiments to compare the transfection efficiencies. Firefly and Renilla luciferase assays were conducted using a Dual-Luciferase® Reporter Assay System (Promega), according to the manufacturer's protocol. Briefly, the cells were washed with PBS and lysed with Passive Lysis Buffer (100 μl/well). The assays for firefly luciferase and Renilla luciferase activities were performed sequentially in a single reaction tube using a 20 μl aliquot of each cell lysate. The cell lysates were mixed with Luciferase Assay Reagent II and firefly luminescence was measured using a luminometer with a dual automatic injector (Lumat LB9507) (PerkinElmer, Boston, MA). Next, the samples were mixed with Stop and Glo reagent, and Renilla luciferase activity was determined as an internal control. Relative luciferase activity was calculated as the ratio of firefly luciferase activity

**Table II-1.** List of primer sequences, product size, PCR conditions, and cycles

| Gene/ Primer | | Sequence (5' to 3') | Product size (bp) | °C (Sec) Denature | Annealing | Extension |
|---|---|---|---|---|---|---|
| *LOX-1* | Forward<br>Reverse | ACTCTCCATggTggTgCCTgg<br>CATTCAgCTTCCgAgCAAggg | 251 | 96 (40) | 60 (30) | 72 (90) |
| *SR-A* | Forward<br>Reverse | gCAgTTCTCATCCCTCTCATTggA<br>ATTCCCATgTCCCTgTggACTgAg | 335 | 96 (40) | 55 (30) | 72 (90) |
| *CD36* | Forward<br>Reverse | gAgACCTgCTTATCCAgAAgAC<br>gACCAACTgTggTAgTAACAgg | 510 | 96 (40) | 55 (30) | 72 (90) |
| *CLA-1* | Forward<br>Reverse | TgATgATggAgAATAAgCCCAT<br>TgACCgggTggATgTCCAggAAC | 696 | 96 (40) | 55 (30) | 72 (90) |
| *CD68* | Forward<br>Reverse | gCCACTCACAgTCCTgCCACC<br>ggACACATTgTACTCCACCgCC | 426 | 96 (40) | 62 (30) | 72 (90) |
| *SR-PSOX* | Forward<br>Reverse | TACACgAggTTCCAgCTCCT<br>gggggCTggTAggAAgTAAA | 154 | 95 (30) | 58 (60) | 72 (60) |
| *CD11b* | Forward<br>Reverse | TCggCggATgAAggAgTTTg<br>CTTTgCACCCggTTCCgTAAg | 756 | 96 (60) | 56 (60) | 72 (120) |
| *CD18* | Forward<br>Reverse | TCgTggACAAgACCgTgCTgC<br>CTACTggTCACCgCgAAgATCg | 421 | 96 (60) | 56 (60) | 72 (120) |
| *GAPDH* | Forward<br>Reverse | gCACCACCAACTgCTTAgCAC<br>gTCTgAgTgTggCAgggACTC | 636 | 95 (30) | 58 (60) | 72 (60) |

to Renilla luciferase activity.

*Statistical analysis*

See Chapter I.

**Results**

*Screening of treated Caco-2 cell basolateral media for effects on LOX-1 expression in THP-1 cells*

In order to examine the effects of the 16 Okinawa foods on TPA-induced *LOX-1* expression, each preparation was separately added to the apical side of Caco-2 monolayers at a concentration of 25% (*v/v*). As shown in Table II-2, the basolateral media from *Artemisia indica* Willd and *Chenopoofium* L. as well as 3 Zingiberaceae plants, *Curcuma aromatica* Salisbury (Wild Turmeric), *Curcuma longa* L. (Turmeric), and *Zingiber zerumbet* Smith, markedly reduced LOX-1 mRNA expression. These 5 preparations were then assayed at a concentration of 5% (*v/v*). The inhibitory rates (IR) of the basolateral media from the 3 Zingiberaceae plants were remarkable (IR > 75%), without any cytotoxicity shown toward either the Caco-2 or THP-1 cells. In contrast, *Saccharum officinarum* L. (sugar cane), *Ipomoea batatas*, Compositae, *Ixeris dentate* Nakai. Cucurbitaceae, *Momordica charantia* L. (balsam pear), and *Luffa cylindrical* Roem (sponge gourd) increased LOX-1 mRNA expression by 1.5- to 2.0-fold.

*Concentration- and time-dependent* LOX-1 *gene suppression by zerumbone*

Several types of substances were found present in the basolateral medium of *Z. Zerumbet*-added differentiated Caco-2 cells and classified into: 1) intact components in *Z. Zerumbet* that penetrated the paracellular pathway or tight junction; 2) their metabolites, including conjugates with glucuronic and sulfuric acids and/or methylated derivatives; 3) chemically degraded products; and 4) secreted factors, including hormones and cytokines from Caco-2 cells in response to stimulation from the homogenate stimuli, as well as other minor components. The active basolateral medium was separated into low molecular (molecular weight; MW < 5,000) and high molecular (MW > 5,000) weight fractions. Only the low molecular fraction had suppressive effects on LOX-1 (data not shown), suggesting that the active constituents of *Z. zerumbet* are low molecular substances. To investigate the active constituents of *Z. zerumbet*, the

**Table II-2** Suppressive effects of the basolateral media from differentiated Caco-2 cells Treated with Okinawan food items on TPA-induced *LOX-1* mRNA expression in THP-1 cells

| Family/species | Part tested | 25% (*v/v*) | | | 5% (*v/v*) | | |
|---|---|---|---|---|---|---|---|
| | | *LOX-1* IR (%) | Caco-2 CV (%) | THP-1 CV (%) | *LOX-1* IR (%) | Caco-2 CV (%) | THP-1 CV (%) |
| Zingiberaceae | | | | | | | |
| *Zingiber zerumbet* Smith | R | 100 | 221 | 72 | 85 | 105 | 97 |
| *Curcuma aromatica* Salisbury | R | 100 | 96 | 100 | 87 | 102 | 98 |
| *Curcuma longa* Linn | R | 100 | 13 | 100 | 76 | 122 | 98 |
| Compositae | | | | | | | |
| *Artemisia indica* Willd | L | 100 | 52 | 100 | 5.0 | 140 | 98 |
| *Gynura bicolor* | L | -21 | 86 | 100 | NT | NT | NT |
| *Ixeris dentate* Nakai | L, ST | -70 | 116 | 100 | NT | NT | NT |
| Chenopodiaceae | | | | | | | |
| *Chenopoofium* L. | R | 87 | 142 | 100 | -2.5 | 99 | 99 |
| Umbelliferae | | | | | | | |
| *Peucedanum japonicum* Thunb | R, ST | 61 | 102 | 100 | NT | NT | NT |
| Liliaceae | | | | | | | |
| *Allium bakeri* | B | 52 | 21 | 100 | NT | NT | NT |
| Spermatochnaceae | | | | | | | |
| *Nemacystis decipiens* Kuckuck | W | 39 | 92 | 100 | NT | NT | NT |
| Monostromataceae | | | | | | | |
| *Monostroma nitidum* Wittrock | W | 6.5 | 95 | 100 | NT | NT | NT |
| Rutaceae | | | | | | | |
| *Citrus deressa* | F | -6.0 | 91 | 100 | NT | NT | NT |
| Cucurbitaceae | | | | | | | |
| *Momordica charantia* L. | F | -45 | 116 | 100 | NT | NT | NT |
| *Luffa cylinadrical* Roem. | F | -46 | 87 | 100 | NT | NT | NT |
| Convolvulaceae | | | | | | | |
| *Ipomoea batatas* | S | -108 | 111 | 100 | NT | NT | NT |
| Gramineae | | | | | | | |
| *Saccharum officinarum* L. | ST | -129 | 165 | 100 | NT | NT | NT |

Plant parts tested B, bulb; F, fruit; L, leaves; S, Stalks; ST, stem; R, root; W, whole part. Caco-2 cells differentiated on a cell culture insert were incubated with the food preparation (5 or 25%, *v/v*) or PBS in the apical side, and incubated at 37° C for 24 h. After incubation, THP-1 cells (1.5×106) were incubated in the collected basolateral medium (1 mL) in a 12-well plate for 30 min, followed by stimulation with TPA (30 nM) for 24 h. Inhibitory effects on *LOX-1* mRNA expression were evaluated by RT-PCR as described in the Materials and methods section. IR; inhibitory rate, NT; not tested. The cell viability (CV) of Caco-2 and THP-1 cells were measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and a trypan blue-dye exclusion test, respectively.

author analyzed ethyl acetate extracts of its homogenate using high-performance liquid chromatography and zerumbone, a major component of *Z. zerumbet*,[136] was detected at a high concentration (64%, *w/w*) (data not shown), and selected

for further study. As shown in Fig. II-1A, TPA-treated THP-1 cells had increased LOX-1 mRNA levels, which were significantly reduced by pretreatment with zerumbone (1-20 μM) in a concentration-dependent manner, with 50% inhibition occurring at a concentration of 9.4 μM. Zerumbone (50 μM) conditioned basolateral media showed significant LOX-1 suppression from 6-24 h (IR = 67.4-82.2%) (Fig. II-1B). The concentration of zerumbone in the 5% (*v*/*v*) homogenate of *Z. Zerumbet* was 130 μM, as quantified by HPLC analysis

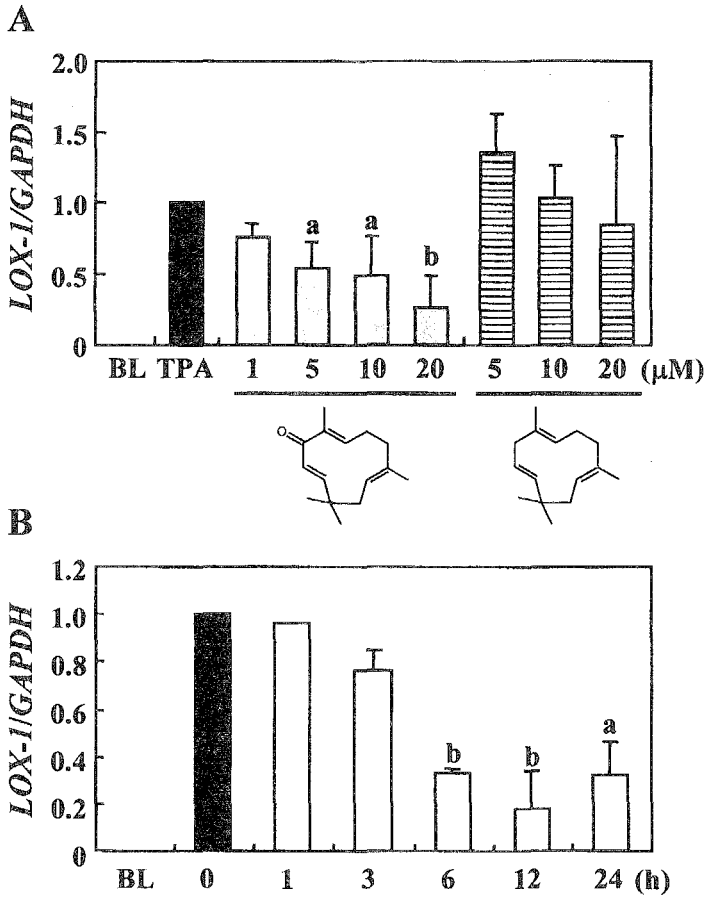


**Fig. II-1** Dose- and time-dependent effects of zerumbone on TPA-induced *LOX-1* mRNA expression in THP-1 cells. (A) Cells ($1.5\times10^6$ cells/ml) were cultured for 30 min with zerumbone (1-20 μM), α-humulene (5-20 μM), or the vehicle, followed by stimulation with TPA (30 nM) for 24 h. (B) Zerumbone at a concentration of 50 μM was added to the apical sides of Caco-2 monolayers for 0, 1, 3, 6, 12, and 24 h. At each time point, basolateral medium was collected and its inhibitory effects toward TPA-induced LOX-1 mRNA expression in THP-1 cells measured. THP-1 cells ($1.5\times10^6$ cells) were incubated in 1 ml of each basolateral medium sample for 30 min. Following that pre-incubation, the cells were treated with TPA (30 nM) for 24 h. At the end of the incubation period, the cells were lysed and *LOX-1* mRNA was analyzed by RT-PCR as described in the Materials and Methods section. *LOX-1* mRNA levels were normalized to the levels of *GAPDH* mRNA. $^a P < 0.05$, $^b P < 0.01$ versus TPA in Student's *t*-test. Data are expressed as the means ± SD of 3 independent experiments.

(data not shown). Further, that concentration used in the Caco-2 assay was the non-lethal maximum, which was determined in preliminary experiments (data not shown). In contrast, α-humulene (5-20 μM), a zerumbone analog lacking the α,β-unsaturated carbonyl group present in zerumbone, did not suppress that expression.

*Quantification of zerumbone in apical and basolateral media of Caco-2 monolayers and intracellular compartments*

As shown in Fig. II-2A, the amount of zerumbone in the apical side medium of the Caco-2 monolayers was time-dependently decreased at a concentration of 50 μM. Further, zerumbone was detected in the basolateral side medium at a concentration of 3.8 μM after 1 h of incubation, which increased to 8.3 μM from 12-24 h (Fig. II-2B). Zerumbone was also detected intracellularly at a rate of 30% of the total incubated amount after 1 h of incubation, after which it decreased to 5.1% after 24 h (Fig. II-2C).

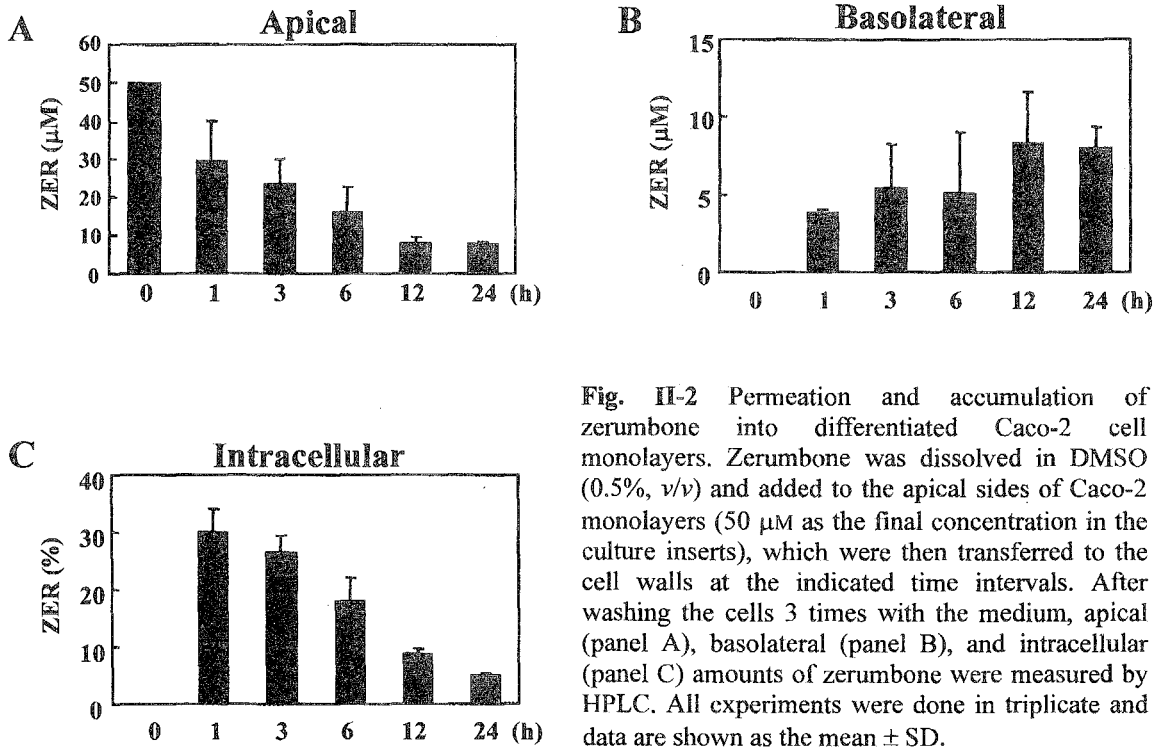


**Fig. II-2** Permeation and accumulation of zerumbone into differentiated Caco-2 cell monolayers. Zerumbone was dissolved in DMSO (0.5%, *v/v*) and added to the apical sides of Caco-2 monolayers (50 μM as the final concentration in the culture inserts), which were then transferred to the cell walls at the indicated time intervals. After washing the cells 3 times with the medium, apical (panel A), basolateral (panel B), and intracellular (panel C) amounts of zerumbone were measured by HPLC. All experiments were done in triplicate and data are shown as the mean ± SD.

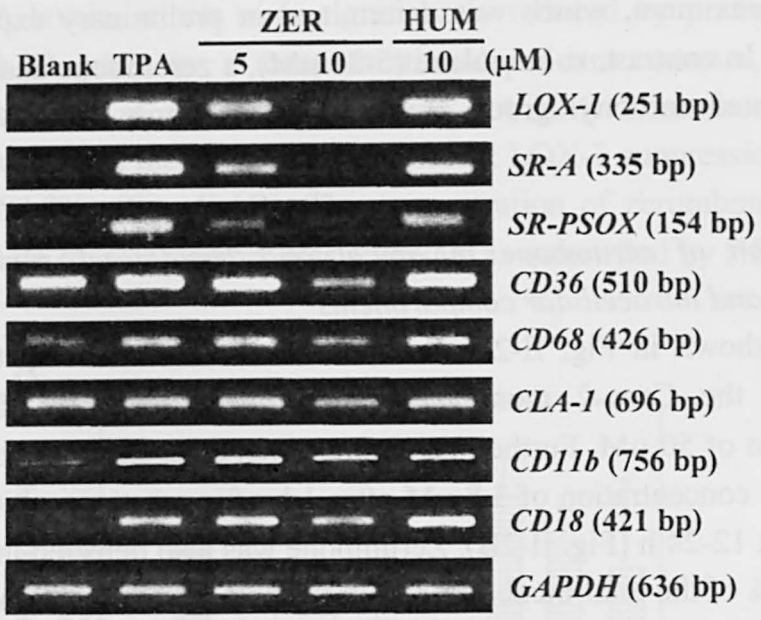


**Fig. II-3** Suppressive effects of zerumbone and its analog on the expression of several different SRs and adhesion molecules. THP-1 cells ($1.5\times10^6$ cells/ml) were incubated with zerumbone (5 or 10 μM), α-humulene (HUM, 10 μM) or the vehicle for 30 min followed by stimulation with TPA (30 nM) for 24 h. mRNA from each sample was quantified by RT-PCR, using *GAPDH* as the standard, as described in the Materials and Methods section. Three independent experiments were performed, with representative data shown.

*Effects of zerumbone and α-humulene on other SRs and expression of adhesion molecules*

Next, the author examined the effects of zerumbone and α-humulene on the mRNA expression of other SRs (*SR-A*, *CD36*, *CD68*, *SR-PSOX*, and *CLA-1*) and adhesion molecules (*CD11b* and *CD18*) using RT-PCR. THP-1 cells were pre-treated with zerumbone (5 or 10 μM), α-humulene (10 μM), or the vehicle, followed by TPA exposure for 24 h. As shown in Fig. II-3, non-treated cells scarcely expressed *SR-A*, *SR-PSOX*, *CD11b*, and *CD18* mRNA, all of which were significantly upregulated following TPA treatment, whereas *CD36*, *CD68*, and *CLA-1* mRNA was expressed in a constitutive manner. Zerumbone (10 μM) abolished the mRNA expression of *SR-A* and *SR-PSOX*, and suppressed that of *CD36* by 85%, whereas that of *CD68*, *CLA-1*, *CD11b*, and *CD18 mRNA* was not markedly changed. On the other hand, α-humulene did not have an effect on the expression of SRs or adhesion molecule mRNA.

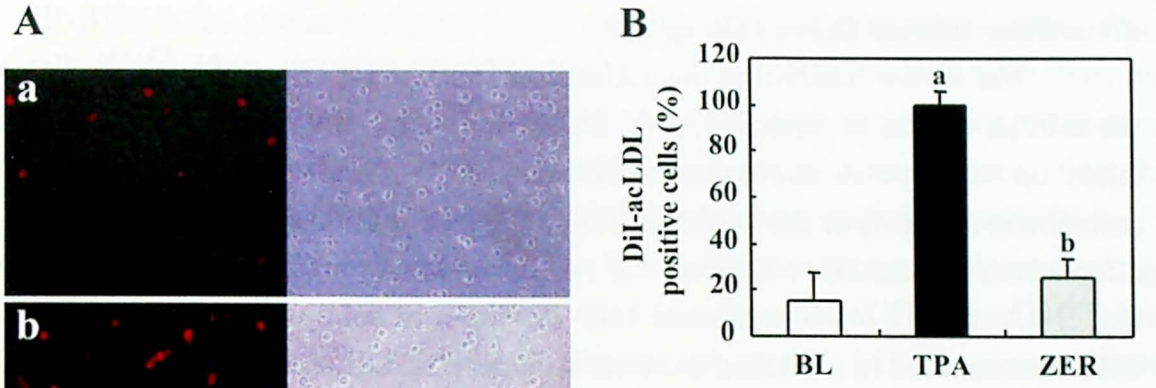


**Fig. II-4** Suppressive effects of zerumbone on uptake of DiI-acLDL. (A) THP-1 cells ($2.5\times10^5$ cells/ml) were incubated with zerumbone (10 μM) or the vehicle for 30 min then stimulated with TPA (30 nM) for 24 h at 37°C. Next, the cells were washed 3 times with PBS and incubated with DiI-acLDL (10 μg/ml) for 4 h at 37°C. DiI-acLDL uptake was assessed by fluorescence photomicroscopy. The experiments were repeated 3 times independently, with 1 representative result for each shown. (a) blank, (b) TPA, (c) zerumbone + TPA. Photographs on the right are bright-field images. (B) Data are expressed as the means ± SD (n = 3). [a] $P < 0.05$ versus blank. [b] $P < 0.05$ versus TPA in Student's *t*-test. Original magnification, × 200.

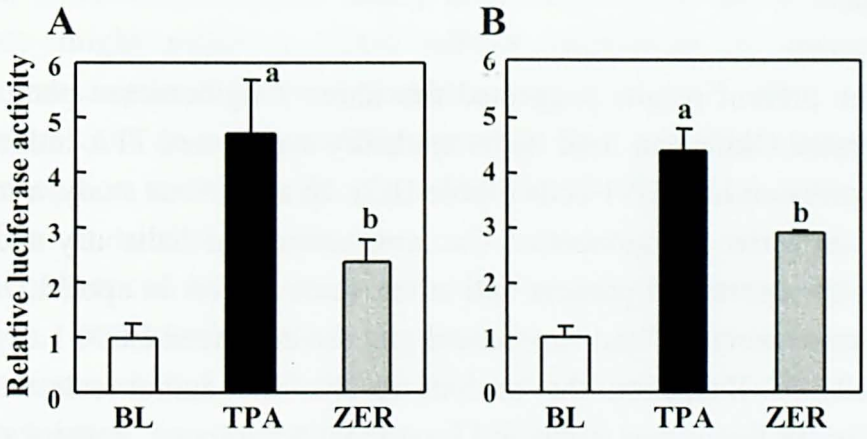


**Fig. II-5** Suppressive effects of zerumbone on AP-1 and NF-κB transactivation. THP-1 cells were transfected with (A) AP-1 or (B) NF-κB promoter-luciferase constructs with a pRL-TK plasmid using lipofectamine for 12 h at 37°C. Next, the transfection reagent was replaced by RPMI medium containing 10% FBS and the cells were incubated for an additional 12 h. The cells were plated in 24-well plates and treated with zerumbone (10 μM) or the vehicle for 30 min then stimulated with TPA (30 nM) for 48 h. Firefly and *Renilla* luciferase activities were determined using a Dual-Luciferase® Reporter Assay System (Promega), according to the manufacturer's protocol. Relative luciferase activity was calculated as the ratio of firefly luciferase activity to that of *Renilla* luciferase. [a] $P < 0.05$ versus blank. [b] $P < 0.05$ versus TPA in Student's *t*-test.

*Zerumbone inhibits DiI-acLDL uptake*

The above results led the author to examine the effect of zerumbone on the cellular uptake of modified LDL. DiI-acLDL is known to be bound to and/or taken up via type A scavenger receptors. THP-1 cells were pre-treated with zerumbone (10 μM) or the vehicle for 30 min, then stimulated with TPA for 24 h, after which DiI-acLDL was observed by fluorescence microscopy. As shown in Fig. II-4A and 4B, undifferentiated THP-1 cells were not positive for DiI-acLDL. TPA treatment led to a marked increase in number of DiI-acLDL-loaded cells and zerumbone (10 μM) dramatically blocked its uptake.

*Zerumbone inhibits AP-1 and NF-κB transactivation*

The expression of *LOX-1* is known to be regulated by several transcriptional factors, including AP-1.[137] To evaluate the mechanism by which zerumbone down-regulates the expression of *SRs*, its inhibitory effects on the TPA-induced activation of AP-1 and NF-κB were examined. TPA-treatment led to a 4.8- and 4.5-fold increase in the transcription activity of AP-1 and NF-κB, respectively, (Figs. II-5AB), while 50% and 30% suppression, respectively, was seen when the cells were pre-treated with zerumbone at a concentration of 10 μM.

**Discussion**

The present results suggested that three Zingiberaceae plants among a group of tested Okinawan food items markedly suppressed TPA-induced LOX-1 mRNA expression in THP-1 cells (Table II-2). In a previous study, a low dose of curcumin, an active component of *Curcuma aromatica* Salisbury and *Curcuma longa* L, was reported to possess anti-atherogenic effects in apoE/LDLR-double knockout mice aortas[138] and suppressed glucose enhanced LOX-1 expression in THP-1 cells.[139] Based on the present results, the author selected *Zingiber zerumbet* Smith for further study and found that zerumbone, a major constituent, suppressed TPA-induced mRNA expression of a number of SRs, including *LOX-1*, *SR-A, SR-PSOX*, and *CD36*, but not that of *CD68* or *CLA-1*, the latter of which is a human ortholog of SR-BI (Fig. II-3). SR-BI and BII play roles in lipid metabolism by mediating cholesterol uptake from bound HDL,[140,141] rather than as long-chain fatty acid transporters. SR-B knockout mice have increased levels of circulating plasma cholesterol via HDL[46,47] and it has been postulated that

SR-BI/II is an anti-atherogenic factor for regulation of cholesterol efflux from cells.[140,142] Thus, in terms of safety it is an advantage that zerumbone does not change the expression of *CLA-1*. Nevertheless, the relevance of suppression of expression by zerumbone *in vitro* in relation to the process of preventing the initiation and progression of atherosclerosis *in vivo* remains to be demonstrated.

Treatment of THP-1 cells with TPA induced a program of macrophage differentiation, as well as marked up-regulation of SR genes[143,144] and *CD11/CD18*.[145] The 5' flanking region of the human LOX-1 gene contains multiple putative binding sites for several transcription factors, including those of AP-1, the GATA family, the STAT family, NF-IL6, Oct-1, CCAAT enhancer-binding proteins, and cyclic AMP response element binding protein.[137] A composite AP-1/ets binding element and the PU.1/Spi-1 binding site are critical for *SR-A* expression during TPA-induced macrophage differentiation,[146,147] and AP-1 is a key regulator of *SR-PSOX* expression in rat aortic smooth muscle cells.[148] Further, protein kinase C activated by TPA is known to stimulate the activities of several classes of transcription factors, including AP-1 and NF-κB.[149] The present results suggest that the inhibitory effects of zerumbone toward TPA-induced SR mRNA expression were partly associated with the suppression of AP-1 and NF-κB activities (Figs. II-5AB). In this study, the expression of *CD36* was not dramatically upregulated by TPA treatment, whereas zerumbone nearly abolished it (Fig. II-3), suggesting that zerumbone might suppress *CD36* mRNA expression by disrupting other unknown transcriptional factor(s), though not by AP-1 or NF-κB.

Murakami *et al.* previously reported that zerumbone suppressed free radical generation as well as the expression of iNOS, cyclooxygenase (COX)-2, and TNF-α in activated leukocytes, and also induced apoptosis in human colorectal cancer cell lines without affecting the growth of normal fibroblasts.[150] In addition, oral administration of zerumbone markedly suppressed dextran sulfate sodium-induced colitis in mice[151] and azoxymethane-induced formation of aberrant crypt foci in rat colons.[134] Takada *et al.* recently showed that zerumbone suppressed NF-κB activation induced by TNF, okadaic acid, cigarette smoke condensate, TPA, and $H_2O_2$, while it also inhibited NF-κB-dependent reporter gene products involved in cell proliferation, anti-apoptosis, and invasion, such as matrix metalloproteinase-9 (MMP-9), COX-2, and ICAM-1.[152] Since COX-2, MMP-9, ICAM-1, and TNF-α are involved in various pathological conditions associated with atherosclerosis,[153-155] zerumbone is expected to have

putative anti-atherogenic, anti-proliferative, and anti-inflammatory effects.

The present results are the first to demonstrate *in vitro* absorption of zerumbone. Zerumbone was added to the apical side (50 μM) of differentiated Caco-2 cells, then after 24 h of incubation, medium collected from the basolateral side was treated with glucuronidase and sulfatase to possibly hydrolyze conjugated zerumbone metabolite(s). Although 8-hydroxy-α-humulene, a putative zerumbone metabolite, was detected by gas chromatography in media from both the apical and basolateral sides, the levels were below the limits of measurement (0.33 μM) (data not shown). The concentration of zerumbone detected in the basolateral side medium after 24 h of incubation was 7.9 μM (Fig. II-2B), which was comparable to a 50% inhibitory concentration of zerumbone (9.4 μM) (Fig. II-1A). Thus, the active component in the *Z. Zerumbet* homogenates was zerumbone, though others may also exist.

Interestingly, α-humulene, a structural analog of zerumbone lacking the α,β-unsaturated carbonyl group, was reported to be completely inactive toward suppressing free radical generation, cell proliferation, and apoptosis induction.[148,152,156] In the present study as well, α-humulene was consistently virtually inactive, suggesting that the α,β-unsaturated carbonyl group is necessary for suppression of SR gene expression (Figs. II-1A, II-3).

In conclusion, the author found that zerumbone permeated into the Caco-2 cell monolayer and showed a distinct ability to suppress the expression of multiple SRs induced by TPA via AP-1 and NF-κB repression, which blocked DiI-acLDL uptake. The present results suggest that this sesquiterpene may be an effective agent to regulate the development of atherosclerosis by preventing foam cell formation. However, additional studies utilizing atherosclerosis-prone animal models are required.

## Chapter III

## Nobiletin, a citrus flavonoid, suppresses phorbol ester-induced expression of multiple scavenger receptor genes in THP-1 human monocytic cells

### Introduction

In Chapter II, the author examined various Okinawan food items and found that the sesquiterpene zerumbone had suppressive effects on TPA-induced SR expression. In this Chapter, in order to extend the knowledge of agents that have physiological potential for the regulation of SR expression, the author investigated the suppressive effects of five different anti-inflammatory food phytochemicals on TPA-induced LOX-1 expression in THP-1. The food phytochemicals used in the present study were selected based on their pronounced anti-inflammatory properties shown in *in vitro* and *in vivo* studies reported previously.[98,157-159] 1'-Acetoxychavicol acetate (ACA) is obtained from the rhizomes of the ethno-medicinal plant *Alpinia galanga* (Zingiveraceae) and is a natural component of a traditional Thai condiment. Auraptene and NOB from citrus fruits, resveratrol, a red wine polyphenol, and (−)-epigallocatechin-3-gallate, the main polyphenol in green tea, were also examined. Further, the author also addressed the underlying molecular mechanisms of the effects of these phytochemicals.

### Materials and Methods

*Materials*

A Nuclear/Cytosol Fractionation Kit® was purchased from BioVision (Mountain View, CA). Antibodies were purchased from the following sources: rabbit anti-phospho-p38 MAPK, rabbit anti-p38 MAPK, rabbit anti-phospho-JNK1/2, rabbit-anti-JNK1/2, rabbit anti-phospho-ERK1/2, rabbit anti-ERK1/2, rabbit anti-phospho-c-Jun (Ser-63), rabbit anti-phospho-c-Jun (Ser-73), rabbit anti-c-Jun, and rabbit antibody horseradish peroxidase (HRP)-linked IgG antibodies came from Cell Signaling Technology (Beverly, MA); mouse anti-c-Fos and goat anti-β-actin antibodies came from Santa Cruz Biotechnology (Santa Cruz, CA); and HRP-conjugated anti-mouse IgG, and anti-goat IgG came from Dako (Glostrup, Denmark). PD98059, SB203580, and

SP600125 were from Calbiochem (La Jolla, CA). All other chemicals were the same as in Chapter I and II unless specified otherwise.

*Cell culture*

See Chapter II.

*RT-PCR*

THP-1 cells at $1.5\times10^6$ were incubated with the test samples or vehicle (0.5% DMSO, *v/v*) for 30 min in 1 ml of RPMI medium with 10% FBS in 12-well plates, followed by stimulation with TPA (30 nM) for the designated periods of time. The suppressive effect of each test sample on SRs mRNA expression was evaluated by RT-PCR, as described Chapter II.

*Reporter assay*

See Chapter II.

*Western blotting*

Cells ($5\times10^6$ cells/3 mL RPMI medium with 10% FBS in a 60-mm dish) were incubated for 30 min with NOB (100 μM) or the vehicle (0.5% DMSO, *v/v*), then stimulated with TPA (30 nM) and collected after 0, 2 and 6 h. The concentration of each agent was determined based on previous cytotoxic experiments (data not shown). Cells were fractionated using a Nuclear/Cytosol Fractionation Kit®, with protease and phosphatase inhibitor cocktails (TaKaRa Bio Shiga, Japan), according to the recommendations of the supplier. The protein concentration in the cytosol fraction was determined using a DC protein assay (Bio-Rad Laboratories, Kyoto, Japan), with γ-globulin used as the standard. Denatured proteins (80 μg) were separated using Sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10 % polyacrylamide gel and transferred onto Immobilon-P Transfer Membranes (Millipore, Bedford, MA). After blocking overnight at 4°C in Block Ace (Dainippon Pharmaceutical, Osaka, Japan), the membranes were first incubated with each antibody at a dilution of 1:1000 [goat anti-β-actin, rabbit anti-phospho-ERK1/2, rabbit anti-ERK1/2, rabbit anti-phospho-p38 MAPK, rabbit anti-p38 MAPK, rabbit anti-phospho-JNK1/2, rabbit anti-JNK1/2, rabbit anti-phospho-c-Jun (Ser-73), rabbit anti-phospho-c-Jun (Ser-63), rabbit anti-c-Jun, and mouse anti-c-Fos (1:500 dilution)]. The second incubation was performed with HRP-conjugated

secondary anti-rabbit IgG, anti-mouse IgG, or anti-goat IgG (1:1000 dilution each). The blots were developed using ECL Western blotting detection reagent (Amersham Biosciences, Buckinghamshire, UK) and the intensity of each band was analyzed using NIH Image. Relative levels of each protein were corrected by employing β-actin as the internal standard.

*Cellular uptake of DiI-acLDL*

See Chapter II.

*Statistical analysis*

See Chapter I.

**Results**

*Effects of selected food phytochemicals on TPA-induced* LOX-1 *mRNA expression in THP-1 cells*

The food phytochemicals used in the present study were selected based on their pronounced anti-inflammatory properties shown in *in vitro* and *in vivo* studies reported previously.[98,157-159] In order to examine their effects on *LOX-1* mRNA expression, THP-1 human monocytic cells were pre-treated with each of the 5 tested food phytochemicals [ACA at 20 μM, auraptene at 100 μM, NOB at 100 μM, (−)-epigallocatechin-3-gallate (EGCG) at 100 μM, resveratrol at 20 μM] or the vehicle, followed by TPA exposure for 24 h. As shown in Fig. III-1A, the vehicle-treated THP-1 cells scarcely expressed *LOX-1* mRNA, as detected by RT-PCR, whereas treatment with TPA highly induced it by 7.3 fold, while NOB attenuated it by 75%. In contrast, ACA, auraptene, EGCG, and resveratrol increased the expression by 1.7- to 3.0-fold.

*Time-dependency of NOB on* LOX-1 *mRNA expression suppression*

As shown in Fig. III-1B, TPA-treated THP-1 cells increased *LOX-1* mRNA levels in a time-dependent fashion from 6 to 24 h. Pretreatment with NOB (0.8–100 μM) for 24 h markedly decreased TPA-induced *LOX-1* mRNA in a concentration-dependent manner. 20 μM have significant suppressive effects (IR > 90%) with the 50% inhibition concentration found to be 2.9 μM.

*NOB inhibited AP-1 transactivation*

The expression of *LOX-1* is regulated by several transcriptional factors, including AP-1[137]. To evaluate the mechanism by which NOB down-regulates the expression of *LOX-1*, the author examined its inhibitory effects on TPA-induced activation of AP-1. TPA-treatment led to a 7-fold increase in AP-1 transcription activity (Fig. III-1C), while a concentration-dependent suppression ranging from 35% to 66% was seen when the cells were pre-treated with NOB in a concentration range of 4–100 μM.

*NOB inhibited MAPK activation*

Activation of the MAPK cascade is well known to contribute to AP-1 transactivation. Therefore, THP-1 cells were pre-treated with NOB (100 μM) or the vehicle for 30 min and then stimulated with TPA for 0, 2, and 6 h. The cytosolic proteins thus obtained were subjected to Western blot analysis to detect

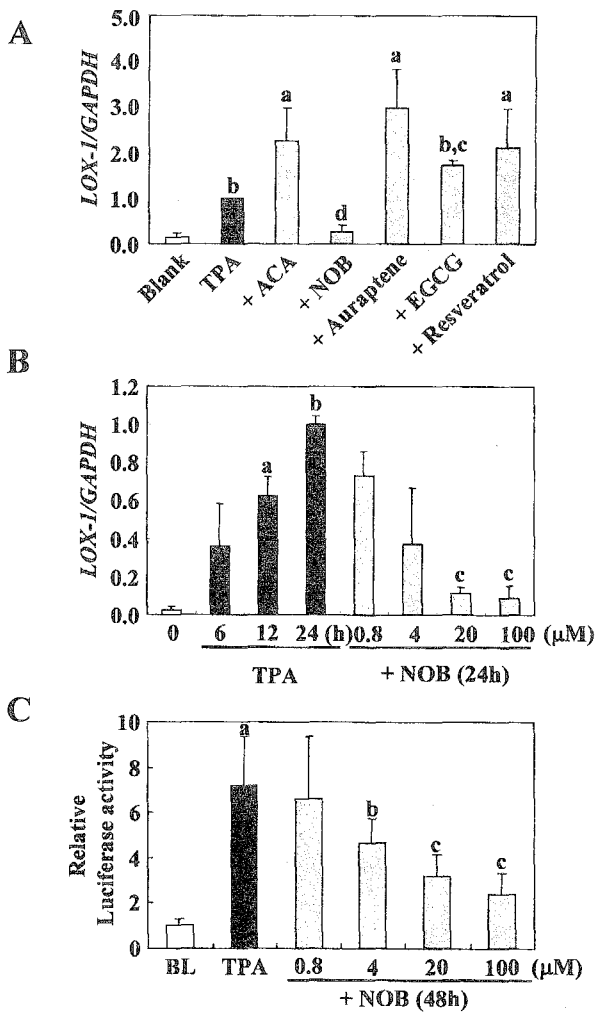


**Fig. III-1** (A) Effects of food phytochemicals on TPA-induced *LOX-1* mRNA expression in THP-1 cells. Cells ($1.5\times10^6$ cells/mL) were incubated separately for 30 min with each of 5 food phytochemicals [ACA (20 μM), NOB (100 μM), auraptene (100 μM), EGCG (100 μM), and resveratrol (20 μM)] or the vehicle, followed by stimulation with TPA (30 nM) for 24 h. At the end of the incubation period, the cells were lysed and *LOX-1* mRNA was analyzed by RT-PCR as described in the Materials and Methods section. *LOX-1* mRNA levels were normalized to the levels of *GAPDH* mRNA. [a] $P < 0.005$, [b] $P < 0.05$ versus blank. [c] $P < 0.005$, [d] $P < 0.01$ versus TPA. Data are expressed as the means ± S.D. of 3 independent experiments. (B) Dose-dependent effect of NOB on TPA induced LOX-1 mRNA expression in THP-1 cells. Cells ($1.5\times10^6$ cells/mL) were cultured with the vehicle for 30 min followed by stimulation with TPA (30 nM) for 6, 12, or 24 h then cultured with NOB at a concentration of 0.8, 4, 20, or 100 μM for 30 min followed by stimulation with TPA (30 nM) for 24 h. At the end of the incubation period, the cells were lysed and *LOX-1* mRNA was analyzed by RT-PCR. *LOX-1* mRNA levels were normalized to the levels of *GAPDH* mRNA. Data are expressed as the means ± S.D. of 3 experiments. [a] $P < 0.01$, [b] $P < 0.001$ versus blank. [c] $P < 0.001$ versus TPA.

both inactive and activated forms of p38 MAPK, JNK1/2, and ERK1/2, using specific antibodies. As shown in Fig. III-2A, following TPA treatment, those kinases were markedly and transiently phosphorylated after 2 h, whereas NOB blocked the TPA-enhanced activation of ERK1/2 and JNK1/2, but not that of p38 MAPK. In addition, the flavonoid significantly reduced c-Jun (Ser-63, but not Ser-73) phosphorylation, while c-Fos expression was not affected. The expression levels of the inactive forms of MAPKs were not significantly changed (data not shown).

*Pharmacological effects on TPA-induced* LOX-1 *gene expression*

To identify the signaling pathways involved in TPA-induced *LOX-1* mRNA expression, THP-1 cells were pre-treated with each of the specific inhibitors for 30 min before exposure to TPA for 12 h. As shown in Fig. III-2B, pretreatment with SB203580 (p38 MAPK inhibitor, 50 μM), SP600125 (JNK1/2 inhibitor, 20 μM), or PD98059 (MEK1/2 inhibitor, 100 μM) significantly suppressed TPA-induced *LOX-1* mRNA expression in a range of 62% to 92%.

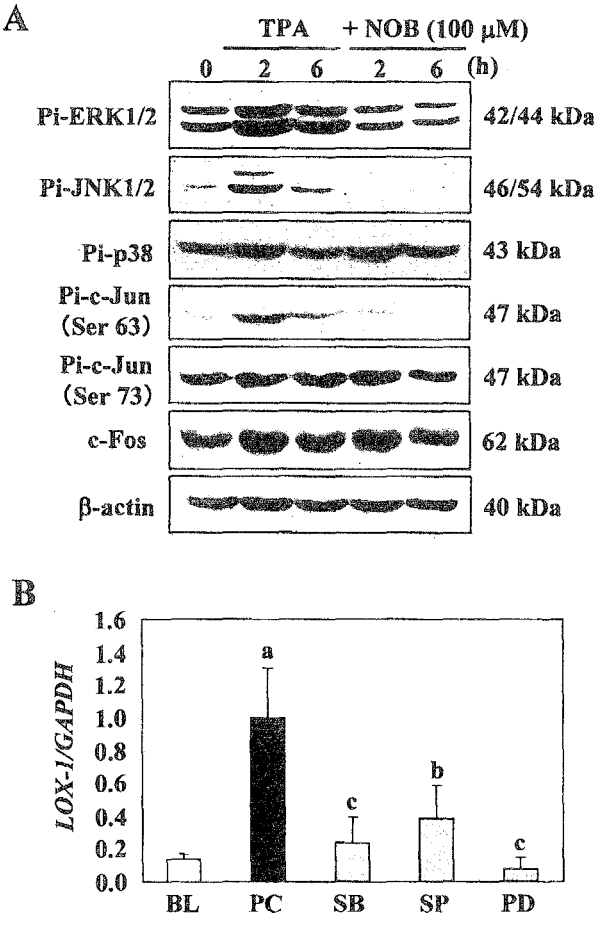


**Fig. III-2** NOB inhibits MAPK activation. (A) Cells ($5\times10^6$ cells/3 mL) were incubated for 30 min with NOB (100 μM) or the vehicle, then stimulated with TPA (30 nM) and collected after 0, 2, and 6 h. The cells were fractionated into cytosolic fractions as described in Materials and methods. Each protein in the cytosolic fraction was subjected to Western blotting with antibodies for phospho-ERK1/2, phospho-JNK1/2, phospho-p38, phospho-c-Jun (Ser-63), phospho-c-Jun (Ser-73), c-Fos, and β-actin. Three independent experiments were performed, with representative results shown. (B) Pharmacological effects toward TPA-induced *LOX-1* gene expression. THP-1 cells were pre-treated separately with each of the specific inhibitors [SB, SB203580 (50 μM); SP, SP600125 (20 μM); PD, PD98059 (100 μM)] for 30 min before exposure to TPA (30 nM) for 24 h. At the end of the incubation period, the cells were lysed and *LOX-1* mRNA was analyzed by RT-PCR. *LOX-1* mRNA levels were normalized to the levels of *GAPDH* mRNA. Data are expressed as the means ± S.D. of 3 experiments. $^{a}P < 0.05$ versus blank, $^{b}P < 0.005$, $^{c}P < 0.001$ versus TPA.

*Pharmacological effects on TPA-induced* LOX-1 *gene expression*

To identify the signaling pathways involved in TPA-induced *LOX-1* mRNA expression, THP-1 cells were pre-treated with each of the specific inhibitors for 30 min before exposure to TPA for 12 h. As shown in Fig. III-2B, pretreatment with SB203580 (p38 MAPK inhibitor, 50 μM), SP600125 (JNK1/2 inhibitor, 20 μM), or PD98059 (MEK1/2 inhibitor, 100 μM) significantly suppressed TPA-induced *LOX-1* mRNA expression in a range of 62% to 92%.

*Effects on other SRs and adhesion molecules expression*

Next, the author examined the effects of NOB on the mRNA expression of other SRs (*SR-A*, *CD36*, *CD68*, *SR-PSOX*, and *CLA-1*) as well as adhesion

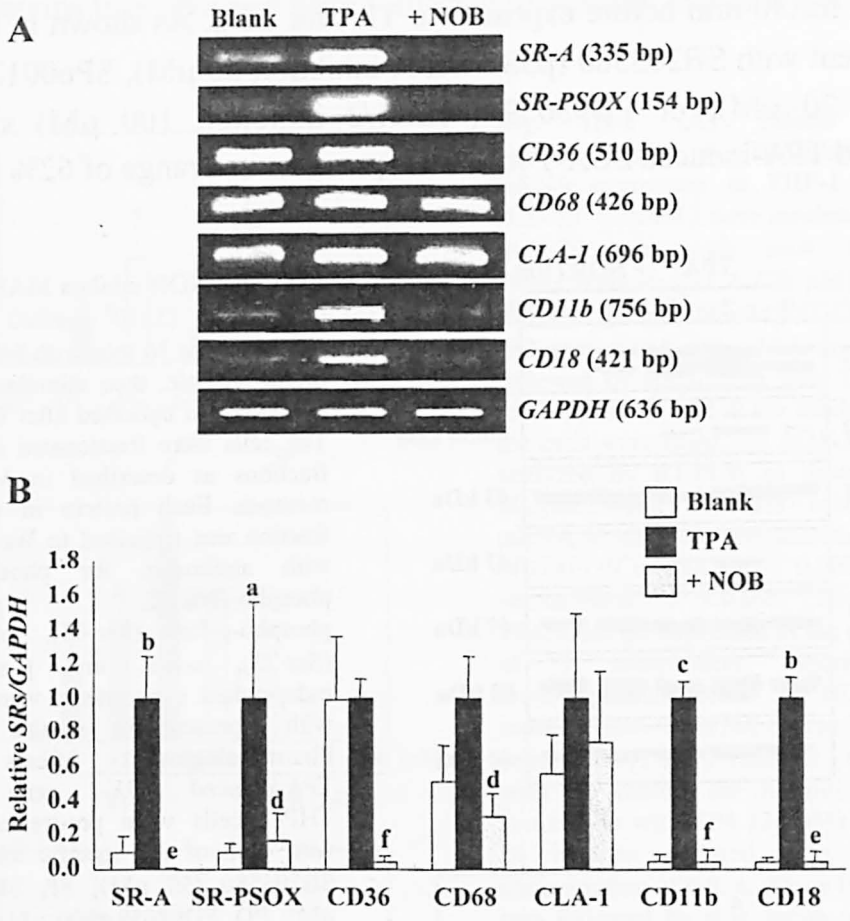


**Fig. III-3** Suppressive effects of NOB on the expression of several different SRs and adhesion molecules. The cells ($1.5\times10^6$ cells/ml) were incubated with 100 μM of NOB or the vehicle for 30 min followed by stimulation with TPA (30 nM) for 24 h. mRNA from each sample was quantified by RT-PCR, using *GAPDH* as the standard, described in Materials and Methods. (A) Three independent experiments were performed, with representative data shown. (B) Each bar represents the mean ± S.D. $^{a}P < 0.05$, $^{b}P < 0.005$, $^{c}P < 0.001$ versus blank. $^{d}P < 0.05$, $^{e}P < 0.005$, $^{f}P < 0.001$ versus TPA.

molecules (*CD11b* and *CD18*) using RT-PCR. THP-1 cells were pre-treated with NOB at 100 μM or the vehicle, followed by TPA exposure for 24 h. As shown in Figs. III-3AB, cells not treated with TPA expressed scant levels of *SR-A*, *SR-PSOX*, *CD11b*, and *CD18* mRNA, while those were significantly upregulated with TPA treatment. In addition, *CD36*, *CD68*, and *CLA-1* mRNA was highly expressed in a constitutive manner in non-treated THP-1 cells and *CD68* mRNA expression was upregulated 2-fold after TPA exposure. NOB abolished the mRNA expression of *SR-A*, *SR-PSOX*, *CD36*, *CD11b*, and *CD18*, while that of *CD68* was decreased to the basal level.

*NOB inhibited DiI-acLDL uptake*

The above results led the author to examine the effect of NOB on cellular uptake of modified LDL. THP-1 cells were pre-treated with 100 μM of NOB or the vehicle for 30 min and then stimulated with TPA for 24 h. Next, the cells were exposed to DiI-acLDL for 4 h at 37°C. No DiI-acLDL uptake by undifferentiated THP-1 cells was observed, whereas TPA treatment led to an increased uptake (Fig. III-4A), which was abolished by NOB.

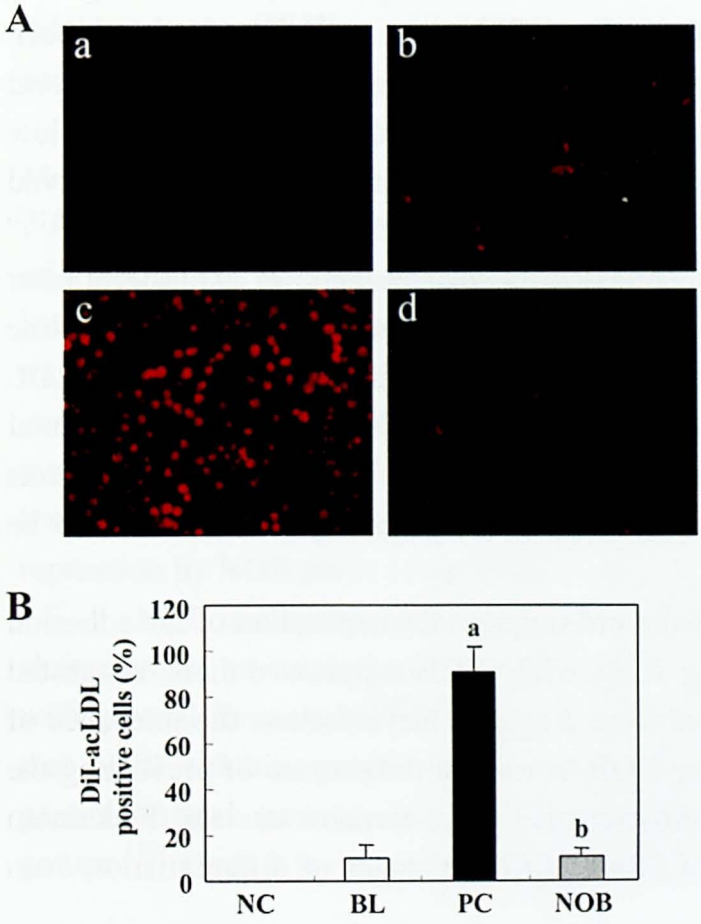


**Fig. III-4** Suppressive effects of NOB on uptake of DiI-acLDL. A: THP-1 cells ($2.5\times10^5$ cells/ml) were incubated with NOB (100 μM) or the vehicle for 30 min and stimulated with TPA (30 nM) for 24 h at 37°C. Then, the cells were washed 3 times with PBS and incubated with DiI-acLDL (10 μg/ml) for 4 h at 37°C. As a negative control, cells were incubated without DiI-acLDL. DiI acLDL uptake was assessed by fluorescence photomicroscopy. The experiments were repeated 3 times independently, with 1 representative result for each shown. (a) Negative control, (b) blank, (c) TPA, (d) NOB + TPA. Approximately 100 cells are shown in each photograph. B: Data are expressed as the means ± S.D. (n = 3). [a] $P < 0.005$ versus blank. [b] $P < 0.005$ versus TPA.

## Discussion

NOB, a polymethoxylated flavone found specifically in citrus fruits,[160] is believed to be a promising anti-inflammatory and anti-tumor promoting agent.[98,161-164] Murakami *et al.* previously reported that NOB acts as a dual inhibitor of $O_2^-$ and NO generation in inflammatory leukocytes, and also showed its suppressive effects toward the formation of inflammatory mediators and tumor promotion in mouse skin.[98,162] Further, NOB has been found to suppress the induction of MMP-7[163] and MMP-9, as well as the release of prostaglandin $E_2$ in rabbit synovial fibroblasts.[164] In addition, Lin *et al.* reported that NOB suppressed the expression of proinflammatory cytokines, including interleukin-1 (IL-1), IL-6, and TNF-α, in mouse macrophages.[161] Those cytokines are involved in various pathological conditions such as atherosclerosis and hypertension,[7] thus NOB may demonstrate a suppressive ability at sites of chronic inflammation.

Other compelling evidence indicates that citrus flavonoids, including NOB, can suppress the hepatic production of cholesterol-containing lipoproteins, thus reducing the total cholesterol concentration in plasma-, and leading to a reduction in the occurrence of cardiovascular disease.[165,166] NOB has been shown to have a hepatic apoB-lowering potential *in vitro*,[166,167] while recent results suggested that it can reduce the circulating concentrations of very low density lipoproteins and LDL in blood, and directly inhibit macrophage-derived foam cell formation at the site of lesion development within vessel walls.[168,169] Whitman *et al.* showed that NOB reduced the accumulation of cholesterol ester mediated by acLDL (a known ligand for SR-A) in the mouse macrophage line J774A.[169] Therefore, it is possible that NOB inhibits the process of acLDL internalization mediated by SR-A, though it did not show an effect toward total and surface SR-A protein expression levels.[169] The action mechanisms underlying the anti-atherogenic effects of NOB described above remain to be clarified.

In Chapter II, zerumbone did not suppress the expression of the adhesion molecules *CD11b* and *CD18* (Fig. II-3), while NOB suppressed them, suggested differences in the action modes of these 2 agents. Nevertheless, the relevance of suppression of their expression by NOB *in vitro* in the process of preventing the initiation and progression of atherosclerosis *in vivo* remains unclear. Tsukamoto *et al.* analyzed the expressions of SRs at different stages of differentiation from

THP-1 monocytes to foam cells, and their results suggested that CD36, CLA-1, and CD68, but not SR-A or LOX-1, play crucial roles in the progression to foam cells from macrophages.[144] The author believes that present results also provide insight into the molecular mechanisms, whose understanding is necessary to explain the anti-atherogenic actions of this flavonoid.

Treatment of THP-1 cells with TPA induces a program of macrophage differentiation and marked up-regulation of SR genes.[143] The 5' flanking region of the human *LOX-1* gene contains multiple putative binding sites for several transcription factors, including those of AP-1, GATA family, STAT family, NF-IL6, Oct-1, CCAAT enhancer-binding proteins, and cyclic AMP response element binding protein.[137] Protein kinase C activated by TPA is known to stimulate the activities of several classes of transcription factors, including AP-1.[149] The present results suggest that the inhibitory effects of NOB toward TPA-induced *LOX-1* expression are partly associated with the suppression of AP-1 activity (Fig. III-1C). This notion is supported by several previous findings, while Kawabata *et al.* recently found that NOB down-regulates MMP-7 expression in HT-29 human colorectal cancer cells via a reduction in AP-1 DNA binding activity.[163] Along a similar line, Sato *et al.* demonstrated that NOB inhibits the invasive activity of TPA stimulated-human fibrosarcoma HT-1080 cells by suppressing AP-1 binding activity.[161]

AP-1 transcriptional activity is dependent on both ERK1/2 and JNK1/2 MAPK-mediated signaling pathways for c-Fos and c-Jun induction, as well as c-Jun phosphorylation. In the present study, NOB inhibited the phosphorylation of JNK1/2 (Fig. III-2A), which presumably results in decreased JNK1/2 activity, leading to a reduced level of c-Jun phosphorylation and blocking of AP-1 transcription activity. Previous reports have shown that ERK1/2 activation contributes to AP-1 activation by Elk-1 phosphorylation, which induces c-Fos synthesis. Although NOB inhibited the phosphorylation of ERK1/2, c-Fos protein levels did not change within 6 h. Thus, it remains unclear whether ERK1/2 repression by NOB plays a role in AP-1 activation.

NOB is a hydrophobic molecule that has 6 methoxyl groups, which is a characteristic associated with a high cellular uptake rate *in vitro* as compared with general flavonoids.[88] In an *in vivo* study as well, Murakami *et al.* showed that NOB exhibits greater localization in the mucosa and muscularis in the gastrointestinal tract as compared to luteolin, a flavonoid with 4 hydroxyl groups.[170] Thus, it is important to examine the ability of NOB to absorb into and

localize within blood vessel walls.

In conclusion, the author found that NOB has a distinct ability to suppress the expression of multiple SRs induced by TPA via AP-1 repression, which blockaded DiI-acLDL uptake. Present results suggest that this flavonoid may be effective as an agent to regulate the development of atherosclerosis. Further studies utilizing atherosclerosis-prone animal models are needed to clarify the present findings.

# Chapter IV

## Suppressive effects of demethylated metabolites of nobiletin on phorbol ester-induced expression of scavenger receptor genes in THP-1 human monocytic cells

### Introduction

In Chapter III, the author demonstrated that NOB markedly reduced the expression of multiple SRs and adhesion molecule genes induced by phorbol ester, such as those of *LOX-1*, *SR-A*, *SR-PSOX*, *CD68*, *CD36*, *CD11b*, and *CD18*, as well as blockaded of DiI-acLDL uptake in THP-1 cells. While the biological activities of NOB have been widely reported,[98,161-164] the metabolic fate of NOB has been studied only recently (Figs. IV-1, 2) The *in vitro* biotransformation of NOB using a rat liver S-9 mixture led to the formation of 3'-demethyl-NOB.[88] Another *in vivo* biotransformation study of NOB in male Sprague-Dawley rats identified the dominant metabolite in rat urine as 3'-demethyl-NOB, with two other mono-demethylated-NOB and two di-demethylated-NOB metabolites reported.[170] Yasuda *et al.* reported that the urinary metabolite of NOB from orally administered rats was 4'-demethyl-NOB, though it is interesting that largely unchanged NOB was also detected in those rat urine samples.[171] Li *et al.* noted that the major metabolite in mouse urine was 4'-demethyl-NOB, whereas 3'-demethyl-NOB was a minor metabolite.[172] Together, these results show that

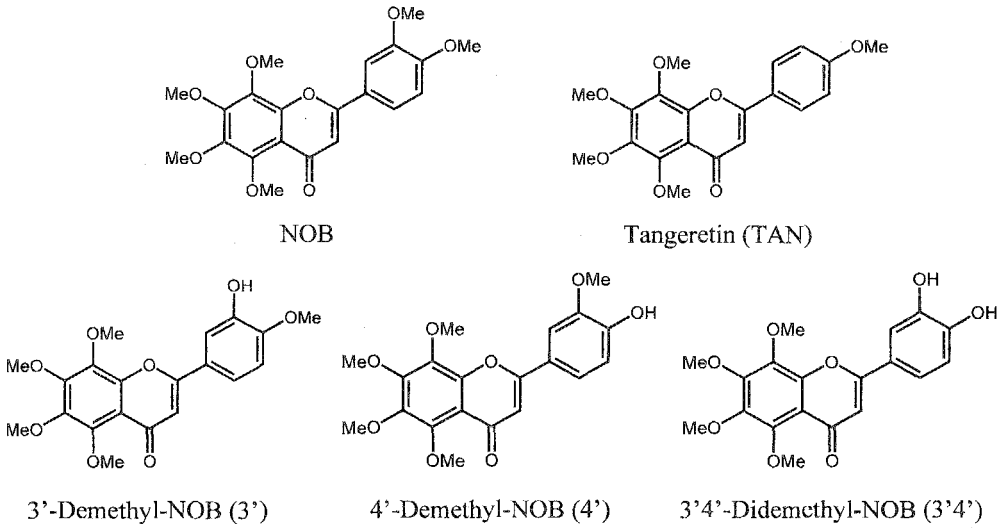


**Fig. IV-1** Structures of NOB, tangeretin, and metabolites of NOB.

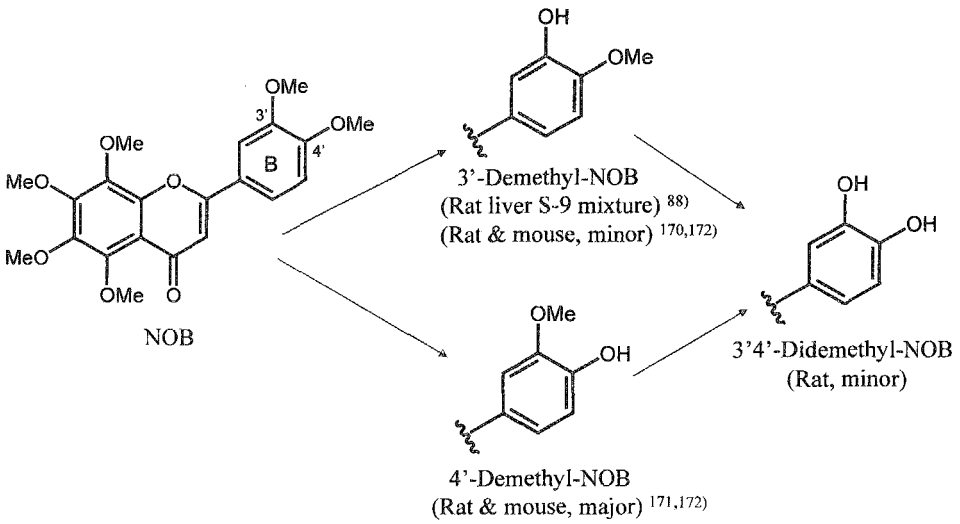


**Fig. IV-2** Metabolic pathways of NOB.

the major NOB metabolites are mono- and didemethylated NOB, though their biological activities are yet to be demonstrated. In this Chapter, the author examined the effects of the NOB metabolites 3'- and 4'-demethyl-NOB, and 3'4'-didemethyl-NOB, as well as that of the NOB analog, tangeretin, on TPA-induced SRs and adhesion molecule mRNA expression in THP-1 cells.

## Materials and Methods

*Chemicals*

NOB metabolites were kindly donated by Prof. Chi-Tang Ho and his colleagues from Rutgers University, NJ. All other chemicals were obtained as described in Chapters I, II, and III. The chemicals structures of the NOB metabolites used in this study are shown in Fig. IV-1.

*RT-PCR*

See Chapter II.

*Reporter assay*

See Chapter II.

*Statistical analysis*

See Chapter II.

## Results

*Effects of metabolites of NOB on TPA-induced* SRs *and adhesion molecule mRNA expression in THP-1 cells*

In order to examine the effects of NOB metabolites on the mRNA expression of the SRs (*SR-A*, *CD36*, *CD68*, *SR-PSOX*, and *CLA-1*) and adhesion molecules (*CD11b* and *CD18*), THP-1 human monocytic cells were pre-treated with 20 μM of each of the metabolites or the vehicle, followed by TPA exposure for 24 h. As shown in Fig. IV-3, vehicle-treated THP-1 cells scarcely expressed *SR-A*, *SR-PSOX*, *LOX-1*, *CD11b*, and *CD18* mRNA as detected by RT-PCR, while those were significantly upregulated following TPA treatment. In addition, *CD36*, *CD68*, and *CLA-1* mRNA was expressed in a constitutive manner in non-treated THP-1 cells. The suppressive effects of 3'-demethyl-NOB on the expression of *SR-A*, *LOX-1*, and *CD36* were weaker than those of the parent NOB. In contrast, 4'-demethyl- and 3'4'-didemethyl-NOB abolished the mRNA expression of *SR-A*, *SR-PSOX*, *LOX-1*, *CD36*, and *CD18* to a greater extent than NOB. In addition, tangeretin at 20 μM had no effect on the expression of any of the SRs or adhesion molecules (Fig. IV-3).

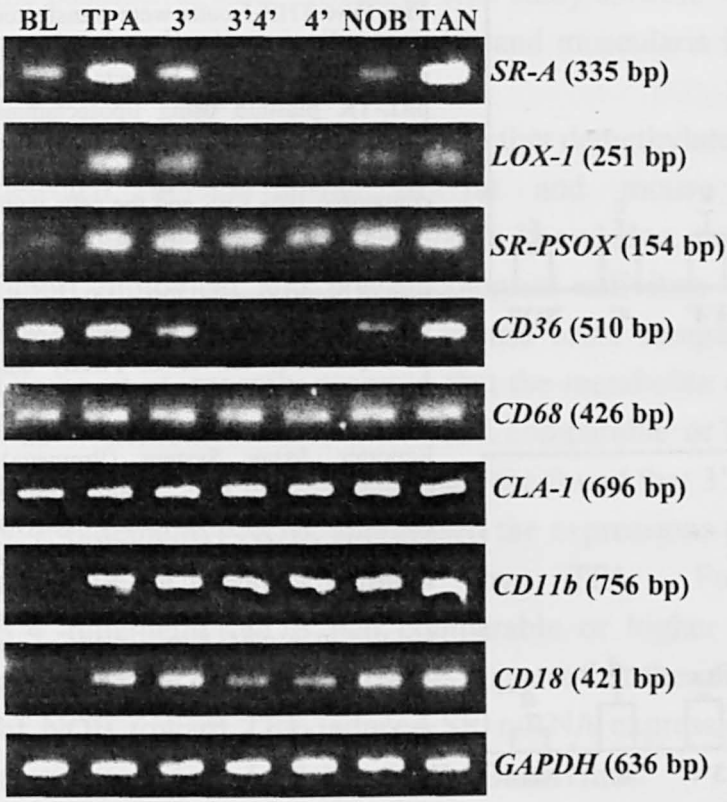


**Fig. IV-3** Effects of metabolites of NOB on TPA-induced *LOX-1* mRNA expression in THP-1 cells. Cells ($1.5\times10^6$ cells/mL) were incubated separately for 30 min with each sample (20 μM), or the vehicle, followed by stimulation with TPA (30 nM) for 24 h. At the end of the incubation period, the cells were lysed and each *SR* mRNA was analyzed by RT-PCR as described in the Materials and Methods section. *SR* mRNA levels were normalized to the levels of *GAPDH* mRNA. Three independent experiments were performed, with representative data shown.

*Metabolites of NOB inhibit AP-1 and NF-κB transactivation*

SR expressions are regulated by several transcriptional factors, including AP-1.[137] To evaluate the mechanism by which metabolites of NOB down-regulate the expression of SRs, we examined the inhibitory effects on TPA-induced activation of AP-1 and NF-κB. TPA-treatment led to a 2.3- and 7.6-fold increase in the transcription activity of AP-1 and NF-κB, respectively (Figs.IV-4AB). 3'-Demethyl-NOB attenuated the transcription activity of AP-1 by 81%, while 4'-demethyl-NOB and 3'4'-didemethyl-NOB suppressed AP-1 significantly to lower than the basal level (Fig. IV-4A). TPA-induced activation of the transcription activity of NF-κB was significantly attenuated by 64%, 85% and 88% by 3'-, 4'-demethyl- and 3'4'-didemethyl-NOB, respectively (Fig. IV-4B).

## Discussion

In this study, the author investigated the suppressive effects of three metabolites of NOB, as well as a structurally related polymethoxyflavone,

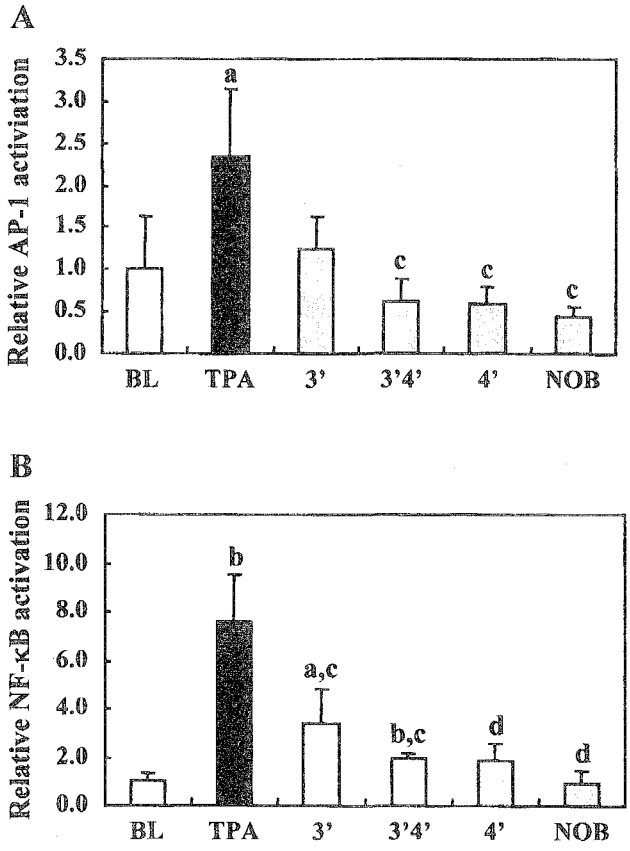


**Fig.** IV-4 THP-1 cells were transfected with (A) AP-1 or (B) NF-κB promoter-luciferase constructs with the pRL-TK plasmid using lipofectamine for 12 h at 37°C. Next, the transfection reagent was replaced by RPMI medium containing 10% FBS and the cells were incubated for an additional 12 h. The cells were plated in 24-well plates and treated with sample (20 μM) or the vehicle for 30 min then stimulated with TPA (30 nM) for 48 h. Firefly and *Renilla* luciferase activities were determined using a Dual-Luciferase® Reporter Assay System (Promega), according to the manufacturer's protocol. Relative luciferase activity was calculated as the ratio of firefly luciferase activity to that of *Renilla* luciferase. [a] $P < 0.05$, [b] $P < 0.01$ versus blank. [c] $P < 0.05$, [d] $P < 0.01$ versus TPA. BL, blank; 3', 3'-demethyl-NOB; 3'4', 3'4'-didemethyl-NOB; 4', 4'-demethyl-NOB.

tangeretin, on the TPA-induced SR and adhesion molecule mRNA expression in THP-1 cells. NOB has been reported to be a promising anti-inflammatory and anti-atherogenic agent,[98,161-164] and shown to have a hepatic apolipoprotein B-lowering potential *in vitro*,[166,167] reduce the circulating concentrations of very low density lipoproteins and LDL in blood, and directly inhibit macrophage-derived foam cell formation at the site of lesion development within vessel walls.[168,169] Whitman *et al.* also showed that NOB reduced the accumulation of cholesterol ester in a mouse macrophage line, J774A,[169] by inhibiting the process of acLDL internalization mediated by SR-A. Further, the author recently reported that NOB markedly reduced the expression of multiple SR and adhesion molecule genes induced by phorbol ester, as well as blockade of DiI-acLDL uptake in THP-1 cells.[173]

It is important to examine the ability of NOB to absorb into and localize within blood vessel walls. NOB is a hydrophobic molecule that has 6 methoxyl groups, which is a characteristic associated with a high cellular uptake rate *in vitro* as compared with general flavonoids. In fact, the intracellular content of NOB was found to be approximately two times higher than that of luteolin, a flavonoid with 4 hydroxyl groups, in differentiated HL-60 cells.[98]Also, NOB tended to accumulate in differentiated Caco-2 cell monolayers and permeated to the basolateral side.[88] In an *in vivo* study as well, we showed that NOB exhibits greater localization in the mucosa and muscularis in the gastrointestinal tract as compared to luteolin.[170]

Recent studies have shown that demethylated metabolites of NOB can be detected in the urine of rat and mouse subjects following NOB consumption.[170-172] To the author's knowledge, the present study is the first to report biological and pharmacological activities of the metabolites of NOB, which were found in rat and mouse urine samples in those previous studies. Okuno *et al.* recently reported that the metabolite of NOB by *Aspergillus niger* was 4'-demethyl-NOB, which had comparable or higher antimutagenic activity than NOB.[174] In the present study, we found that 3'-demethyl-, 4'-demethyl- and 3'4'-didemethyl-NOB, suppressed the expressions of multiple SRs and adhesion molecule genes induced by TPA. Further, 4'-demethyl- and 3'4'-didemethyl-NOB had comparable or higher suppressive effects than the parent NOB. The present results suggest that the inhibitory effects of metabolites of NOB toward TPA-induced SR mRNA expression are partly associated with the suppression of AP-1 and NF-κB activities.

The major metabolite in rat and mouse urine following NOB consumption has been identified as 4'-demethyl-NOB, whereas 3'-demethyl-NOB is a minor metabolite.[171,172] Li *et al.* proposed that methyltransferase enzymes in mice easily remove the methyl groups on the B-ring of NOB, particularly the 4'-methyl group.[172] Nielsen *et al.* reported *in vivo* and *in vitro* biotransformation of the related polymethoxyflavonoid, tangeretin, and the main metabolite was the demethylated compound at the 4'-position of the B-ring in those studies.[175,176] From those results, it is thought that the methoxy group at the 4'-position is subject to demethylation much more than those at other positions. Further, studies of the structure-activity relationships of flavonoids have shown that the presence of the *ortho*-catechol group (3',4'-OH) in the B-ring is a determinant for high antioxidant and anti-proliferative capabilities.[177-179] The present results suggest that demethylation at the 4'-position of the B-ring contributed to the stronger suppressive effects on TPA-induced SR mRNA expression.

Free hydroxyl groups after demethylation may be conjugated by conjugation enzymes such as glucuronic acid or sulfates. Consequently, the polarity and hydrophilicity of the subsequently formed molecules, such as 4'-demethyl-NOB glucuronate or sulfate, are significantly increased and the tendency of these molecules to be easily absorbed and excreted is greatly increased. Shimoi *et al.* reported that luteolin was converted into glucuronides while passing through the intestinal mucosa, and they also detected its conjugates and methylated conjugates in rat plasma after dosing.[180] In addition, Mochizuki *et al.* speculated that circulating conjugates of quercetin pass through the endothelium to reach vascular smooth muscle cells and exert their biological effects in the blood vessels during inflammation, followed by deconjugation of the conjugates.[181] Nevertheless, identification of conjugated demethyl-NOBs or these metabolites in mouse or rat plasma remain to be clarified.

In conclusion, the present results suggest that the NOB metabolites, 4'-demethyl-NOB and 3'4'-didemethyl-NOB are active principles with a high potential to prevent arteriosclerosis *in vivo*.

## Summary

Unregulated uptake of ox-LDL via macrophage SRs such as LOX-1 is a key event in atherosclerosis. Thus, suppressants of SRs are considered to be beneficial for chemotherapy strategies, though studies performed for the identification of such compounds are limited. ROS are considered to play important roles in the onset of a variety of diseases including atherosclerosis. Reactive molecular species including $O_2^-$ rapidly convert the LDL to ox-LDL. Thus, the suppression of excessive $O_2^-$ and NO generation is effective for prevention of oxidative stress-related diseases.

In order to more accurately determine the *in vivo* physiological activities of food items and their components, their bioavailability and metabolism in the gastrointestinal tract must be considered, because most plant secondary metabolites are scarcely absorbed, metabolized, or chemically decomposed there. Moreover individual nutrients and interactions with other nutrients must be taken into account. However, only a limited number of *in vitro* methods for precisely investigating small intestinal absorption and gut wall extraction have been reported.

The author established a novel assay system to reflect *in vivo* anti-oxidative constituents using differentiated Caco-2 cells (Chapter I). When exposed to differentiated Caco-2 cells, five different food preparations exhibited a wide range of antioxidant activities, cytotoxicity, and effects on the tight junction, suggesting that the present assay system is appropriate and reliable for determination of the anti-oxidative efficacy of dietary phytochemicals *in vivo*. Experimental results also showed that a carrot preparation was promising, as the basolateral medium from Caco-2 cells exposed to it highly suppressed NO generation at relatively low concentrations. However, the author was not able to precisely identify the active component present in the carrot preparation-conditioned basolateral medium.

In Chapter II, the author focused on Okinawa food items, because the residents of Okinawa are known for having the greatest longevity in Japan and a low rate of death from cardiovascular disease. Sixteen traditional food items were screened for their suppressive effects on TPA-induced SR expression in THP-1 cells. Three Zingiberaceae plants markedly reduced LOX-1 mRNA expression and *Zingiber zerumbet* Smith was selected for further study. Its major constituent, zerumbone, suppressed TPA-induced SRs, including LOX-1, SR-A,

SR-PSOX, and CD36, but not that of CD68 or CLA-1, leading to a blockade of DiI-acLDL uptake, while it also inhibited the transcriptional activities of AP-1 and NF-κB. In addition, α-humulene, a structural analog of zerumbone, was inactive, suggesting that the α,β-unsaturated carbonyl group present in zerumbone is necessary for suppression of SR gene expression. These results demonstrated for the first time *in vitro* absorption of zerumbone.

In Chapter III, the suppressive effects of five selected food phytochemicals on TPA-induced LOX-1 expression were examined. NOB, a citrus polymethoxylated flavone, showed notable suppressive effects in dose-, and time-dependent manners. Additionally, the author investigated the molecular mechanism underlying the suppression of SR gene expression. NOB also suppressed the phosphorylation of ERK1/2, JNK1/2, and c-Jun (Ser 63), thereby inhibiting the transcriptional activity of AP-1. Further, NOB attenuated the expression of SR-A, SR-PSOX, CD36, and CD68 mRNA, but not that of CLA-1, leading to the blockade of DiI-acLDL uptake. NOB also suppressed the adhesion molecules CD11b and CD18, while zerumbone did not (Chapter II), suggesting differences in the action modes of these 2 agents.

Next, the author examined the effects of the NOB metabolites, 3'- and 4'-demethyl-NOB, and 3'4'-didemethyl-NOB, as well as an NOB analog, tangeretin, on TPA-induced SR and adhesion molecule mRNA expression in THP-1 cells (Chapter IV). 3'-Demethyl-, 4'-demethyl-, and 3'4'-didemethyl-NOB suppressed the expression of multiple SR and adhesion molecule genes induced by TPA. Further, 4'-demethyl- and 3'4'-didemethyl-NOB had comparable or higher suppressive effects than the parent NOB. These results suggest that the inhibitory effects of NOB metabolites toward TPA-induced SR mRNA expression are partly associated with the suppression of AP-1 and NF-κB activities. To the author's knowledge, this is the first report of biological and pharmacological activities of NOB metabolites, which have been found in rat and mouse urine following NOB administration.

In conclusion, zerumbone and NOB remarkably suppressed TPA-induced SRs in THP-1 cells by targeting the transcription step. Together with previous findings, these agents are indicated to be promising potential phytochemicals for regulating atherosclerosis with reasonable action mechanisms. Nevertheless, additional studies utilizing atherosclerosis-prone animal models are needed to clarify these results.

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## List of publications

### Original articles

1. Eguchi, A., Murakami, A., Ohigashi, H. (2005) Novel bioassay system for evaluating anti-oxidative activities of food items: Use of basolateral media from differentiated Caco-2. *Free Radic. Res.*, **39**, 1367-1375.

2. Eguchi, A., Murakami, A., Ohigashi, H. (2006) Nobiletin, a citrus flavonoid, suppresses phorbol ester-induced expression of multiple scavenger receptor genes in THP-1 human monocytic cells. *FEBS Lett.*, **580**, 3321-3328.

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